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Disk Diffusion Antimicrobial Susceptibility Testing of Members of the Family *Legionellaceae* Including Erythromycin-Resistant Variants of *Legionella micdadei*

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Disk diffusion antimicrobial susceptibility testing of members of the family *Legionellaceae* was accomplished on buffered charcoal yeast extract agar by allowing the bacteria to grow for 6 h before placement of the disks, followed by an additional 42-h incubation period before the inhibitory zones were measured. This system was standardized by comparing the zone sizes with the MICs for 20 antimicrobial agents of nine bacterial strains in five *Legionella* species and of 19 laboratory-derived, erythromycin-resistant variants of *Legionella micdadei*. A high, linear correlation between zone size and MIC was found for erythromycin, trimethoprim, penicillin, ampicillin, carbenicillin, cephalothin, cefamandole, cefoxitin, moxalactam, chloramphenicol, vancomycin, and clindamycin. Disk susceptibility testing could be employed to screen *Legionella* isolates for resistance to any of these antimicrobial agents, of which only erythromycin is known to be efficacious in the treatment of legionellosis. With selected antibiotics, disk susceptibility patterns also appeared to accurately identify to the species level the legionellae. The range of the MICs of the legionellae for rifampin and the aminoglycosides was too small to determine whether the correlation of zone size with MIC was linear. However, laboratory-derived, high-level rifampin-resistant variants of *L. micdadei* demonstrated no inhibition zone around the rifampin disk, indicating that disk susceptibility testing would likely identify a rifampin-resistant clinical isolate. Of the antimicrobial agents tested, the only agents for which disk susceptibility testing was definitely not possible on buffered charcoal yeast extract agar were oxacillin, the tetracyclines, and the sulfonamides.

The antimicrobial susceptibilities of the legionellae have previously been examined by agar dilution testing, and the susceptibility patterns found by a number of investigators have been consistent within each *Legionella* species (6, 9, 11, 17, 20, 22, 23). However, since relatively few *Legionella* strains have been tested, it is possible that susceptibility patterns are not universal within each species. Furthermore, resistance of the legionellae to erythromycin, rifampin, or other antimicrobial agents could develop at any time, particularly among strains isolated from patients receiving antibiotic therapy. Since the legionellae grow only slowly on special media, agar dilution susceptibility testing is not a routine procedure for the clinical microbiology laboratory. Thus, there is a need for a simple system by which the legionellae can at least be screened for antimicrobial susceptibility in the clinical laboratory.

We developed a disk diffusion susceptibility testing system for the legionellae and compared the zone sizes obtained with the MIC for each antimicrobial agent. Since the differences in the MICs of strains within the same species of *Legionella* are small, the disk susceptibility system was standardized with representative strains in different species. In addition, we obtained *Legionella* strains with varying degrees of erythromycin resistance to extend the range of the MICs to this antibiotic of the organisms examined. It was found that disk diffusion testing is feasible with the legionellae for a number of antimicrobial agents. In addition, disk susceptibility testing may be useful for preliminary identification of *Legionella* isolates to the species level.

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MATERIALS AND METHODS

Media. Buffered charcoal yeast extract (BCYE) agar was made as previously described (18). For disk diffusion testing, 60 ml of molten agar was dispensed into 150-mm petri dishes on a level surface to ensure uniform depth of the agar. Buffered yeast extract broth (BYEB) was prepared as described previously (19), except that 10 g of acetamidoaminoethanesulfonic acid (Research Organics, Cleveland, Ohio) per liter was added.

Bacteria. The following nine strains of five species of *Legionella* were employed: *Legionella pneumophila* serogroup 1 Philadelphia 1; *Legionella micdadei* EK (ATCC 33204), CR, and LD; *Legionella bozemanii* WIGA and MI-15; *Legionella dumoffii* Tex-KL and NY-23; and *Legionella gormanii* LS-13. The three *L. micdadei* strains were orginally isolated by us from patients (16), whereas the remaining *Legionella* strains were obtained from the Centers for Disease Control (Atlanta, Ga.). All strains were passed multiple times on BCYE agar. Stock cultures for disk susceptibility testing were made by passing each strain once in BYEB; portions of this passage were stored frozen at −70° in BYEB without iron or cysteine, to which 10% heat-inactivated newborn calf serum (GIBCO Laboratories, Grand Island, N.Y.) was added.

To obtain *L. micdadei* strains with increased MICs to erythromycin, resistant variants were selected by a single passage on medium containing erythromycin. A portion of the stock culture of strain EK was thawed, and 0.1 ml was plated on BCYE agar. The growth on these plates was harvested into sterile 0.85% saline, and the turbidity of this suspension was adjusted to match a McFarland number 3 standard by the addition of saline. A 0.1-ml (containing

about 10^8 bacteria) was plated on BCYE agar containing 2.5, 5.0, or 50 μg of erythromycin lactobionate (Abbott Pharmaceuticals, North Chicago, Ill.) per ml. After 1 to 2 weeks of incubation, individual colonies were picked and passed to erythromycin-free BCYE agar. The growth from these plates was employed to make stock cultures as described above. Rifampin-resistant variants were obtained by the same procedure with BCYE agar containing 10 μg of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml. All erythromycin- or rifampin-resistant strains were subjected to autoclaving before disposal by incineration.

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 were utilized as control organisms.

MIC determinations. The MIC of each strain, including the erythromycin- and rifampin-resistant variants of EK, was determined on BCYE agar with a Steers replicator as described previously (17). The MIC for each antimicrobial agent was determined at least twice for each *Legionella* strain, and the geometric mean MIC was calculated.

Disk diffusion susceptibility testing. Portions of the stock cultures of each bacterial strain were grown in BYEB for approximately 16 h, and the resulting growth was adjusted with BYEB to an optical density of 0.025 to 0.03 at 650 nm with a "Junior" spectrophotometer (Coleman Instruments, Inc., Maywood, Ill.) and a cuvette with a 1.5-cm light path. Suspensions at this optical density contained between 5×10^7 and 1×10^8 viable bacteria per ml. This suspension was spread on a BCYE agar plate with a cotton swab, and the plate was incubated at 37°C for 6 h. At this time the antimicrobial disks were dropped onto the agar, and the plate was returned to the incubator. Zone sizes were read after an additional 42 h of incubation. Zone sizes for each strain were determined at least twice for all antimicrobial agents, and the average zone sizes were calculated. For each antimicrobial agent, the average zone size of each strain was plotted against the $\log_2$ geometric mean MIC of that strain, and the regression line was determined by the least-squares method (21).

Commercially available antimicrobial disks (BBL Microbiology Systems, Cockeysville, Md.) were employed, except for trimethoprim. Disks for this agent were made by diluting a 10-mg/ml stock solution of trimethoprim (Burroughs Wellcome Co., Research Triangle Park, N.C.) in 0.05 N lactic acid to the appropriate concentration with sterile water, applying 20 μl onto blank disks (BBL), and allowing the disks to dry overnight.

RESULTS

The disk inhibitory zone sizes found for the *S. aureus* and *E. coli* control organisms with BCYE agar were within the range of the published zone sizes for Mueller-Hinton agar (2) only for clindamycin, vancomycin, chloramphenicol, tetracycline, streptomycin, trimethoprim-sulfamethoxazole, and nalidixic acid (Table 1). For the remaining 15 antimicrobial agents tested, the zone sizes for one or both control strains differed between the two media, indicating that disk diffusion susceptibility testing on BCYE agar must be independently standardized.

The zone sizes are plotted against the MICs of the nine *Legionella* strains for the penicillins (Fig. 1). The slopes of the regression lines were very similar for pencillin, ampicillin, and carbenicillin ($b = -0.20$ to -0.26), and the regression coefficients were high for each of these antibiotics. In contrast to penicillin G, ampicillin, and carbenicillin, there was not a linear relationship between the zone sizes and the MICs of the legionellae for oxacillin (Fig. 1D). Rather, there were no zones of inhibition around the oxacillin disk with the relatively resistant *L. pneumophila* and *L. dumoffii* strains, whereas the relatively sensitive bacteria, *L. micdadei*, *L. bozemanii*, and *L. gormanii*, produced zones varying from 12 to 23 mm.

The slopes of the regression lines were essentially the same for cephalothin ($b = -0.18$) and cefamandole ($b = -0.23$), and the correlation between MIC and zone size was very good for both of these antibiotics (Fig. 2A and B). The slopes of the regression lines for cefoxitin ($b = -0.13$) and moxalactam ($b = -0.22$) were similar, although the correlation of MIC with zone size was not quite as good (Fig. 2C and D) as with cephalothin and cefamandole.

The MICs and zone sizes with erythromycin of the naturally occurring *Legionella* strains are shown in Fig. 3. The regression coefficient was reasonably high considering the relatively small range of the MICs of these strains.

Nine strains of *L. micdadei* EK which grew on BCYE agar containing 2.5 μg of erythromycin per ml were recovered and found to have MICs to erythromycin ranging from 1.0 to 4.0 μg/ml. The range of the MICs of the 10 strains which grew at 5.0 μg of erythromycin per ml was from 5.0 to 32.0 μg/ml. The MICs and zone sizes for erythromycin of each of these resistant variants was determined on two separate occasions, and the geometric mean MICs and mean zone sizes for the two determinations are plotted in Fig. 4, along with the data for the naturally occurring legionellae from Fig. 3. The slope of the regression line for the erythro-

TABLE 1. Disk zone inhibition sizes of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 for 22 antimicrobial agents on BCYE and Mueller-Hinton agars

Antimicrobial agent	Disk content[a]	Zone size (mm) for following organism on indicated agar:			
		S. aureus		*E. coli*	
		BCYE[b]	Mueller-Hinton[c]	BCYE[b]	Mueller-Hinton[c]
Penicillin G	10	41.3	26–37		
Oxacillin	1	26.8	17–22[d]		
Ampicillin	10	31.1	24–35	22.6	15–20
Carbenicillin	100			20.8	24–29
Cephalothin	30	38.3	25–37	15.7	18–23
Cefamandole	30	38.0	28–34	30.0	24–31
Cefoxitin	30	29.8	23–28	26.4	23–28
Erythromycin	15	25.9	22–30	6.0	8–14
Clindamycin	2	25.1	23–29		
Vancomycin	30	18.9	15–19		
Chloramphenicol	30	25.0	19–26	26.7	21–27
Tetracycline	30	26.2	19–28	18.0	18–25
Streptomycin	10	14.8	14–22	14.4	12–20
Kanamycin	30	16.1	19–26	15.3	17–35
Gentamicin	10	13.5	19–27	12.1	19–26
Tobramycin	10	12.6	19–29	10.5	18–26
Amikacin	30	13.3	20–26	16.1	19–26
Colistin	10			9.2	11–15
Sulfisoxazole	250	27.4	24–34	6.0	18–26
Trimethoprim-sulfamethoxazole	1.25-24.5	24.5	24–32	24.0	24–32
Nitrofurantoin	300	17.8	20–24	19.5	21–26
Nalidixic acid	30			24.8	23–28

[a] Contents shown in micrograms except that of pencillin G, which is shown in units.
[b] Values are the mean of two determinations.
[c] Data obtained from reference 2.
[d] Methicillin, 5-μg disk.

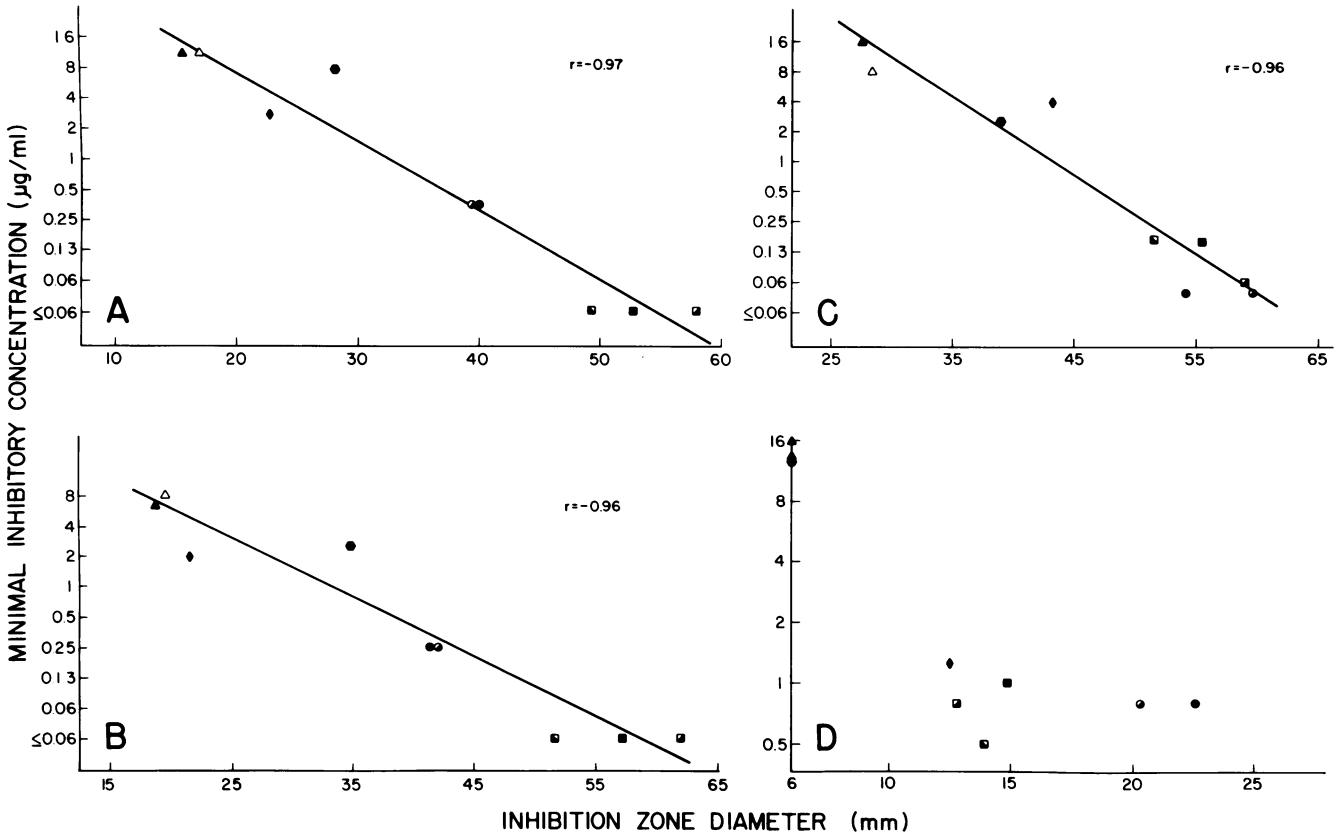

FIG. 1. Mean disk inhibition zone sizes and geometric mean MICs obtained with penicillin G (A), ampicillin (B), carbenicillin (C), and oxacillin (D) for nine strains of *Legionella*: *L. pneumophila* serogroup 1 Philadelphia 1 (●); *L. micdadei* EK (◧), CR (◪), and LD (■); *L. bozemanii* WIGA (◑) and MI-15 (●); *L. gormanii* LS-13 (◆); and *L. dumoffii* Tex-KL (△) and NY-23 (▲). The regression line of zone size on MIC is shown along with the regression coefficient (*r*).

mycin-resistant variants ($b = -0.20$) differed little from the slope when the three naturally occurring *L. micdadei* isolates were included ($b = 0.21$) or from the slope when all natural *Legionella* strains were included ($b = -0.23$). Three variant strains which grew at 50 μg of erythromycin per ml demonstrated no zone of inhibition around the 15-μg erythromycin disk.

Trimethoprim disks were made containing 1.25, 5.0 and 15.0 μg per disk. With all three concentrations, the relation of zone size to MIC for the legionellae was linear and the regression coefficient was high. However, with the 1.25-μg disks, there was either no zone of inhibition or very small zones around the disk with the three *L. micdadei* strains. With the 15-μg disk, the zones with the *L. dumoffii* and *L. gormanii* strains were inconveniently large. The results with a 5-μg disk are shown in Fig. 5. It appears that the commercially available trimethoprim-sulfamethoxazole (1.25 μg-23.7 μg) disk could also be employed (Fig. 6). However, we obtained very erratic results for the legionellae with a 250-μg sulfisoxazole disk (data not shown). The problem is exemplified by the results shown in Table 1; sulfisoxazole yielded a zone for *S. aureus* which was the same as that obtained on Mueller-Hinton agar, but there was no zone with the control *E. coli*. The latter was to be expected since BCYE agar contains *para*-aminobenzoic acid (17). However, it was not clear why a reasonable zone was observed with *S. aureus* and why the results with the legionellae varied erratically. In view of the irregular results, testing of the legionellae with the trimethoprim-sulfamethoxazole disk may prove to be less reliable than testing with a trimethoprim disk.

The regression line for clindamycin had a high correlation coefficient (Fig. 7). It is of interest that two strains within the same genus (WIGA and MI-15) demonstrated divergent MICs for clindamycin which were reflected in the inhibitory zone sizes observed. The correlation coefficient for vancomycin was also high when only those strains with definite MICs were considered (Fig. 8). However, those organisms with MICs greater than 32 μg/ml did not appear to fall on the regression line. The correlation of zone sizes and MICs was somewhat lower for chloramphenicol (Fig. 9), probably because the range of the MICs of the legionellae was too small to see a better correlation.

The ranges of the MICs of the *Legionella* strains to the aminoglycosides (streptomycin, gentamicin, tobramycin, amikacin) and rifampin were too small to determine the correlation with zone sizes. The rifampin-resistant *L. micdadei* variants isolated from BCYE agar containing 10 μg of rifampin per ml all had MICs of over 100 μg/ml. There was no zone of inhibition around the 5-μg rifampin disk for any of these resistant strains.

The only antibiotics, in addition to oxacillin, for which there was poor correlation of the MICs and zone sizes were tetracycline and doxycycline. We have already shown that there are inhibitors of the tetracyclines in BCYE agar which prevent the determination of the MICs of the legionellae for this class of antibiotics (17).

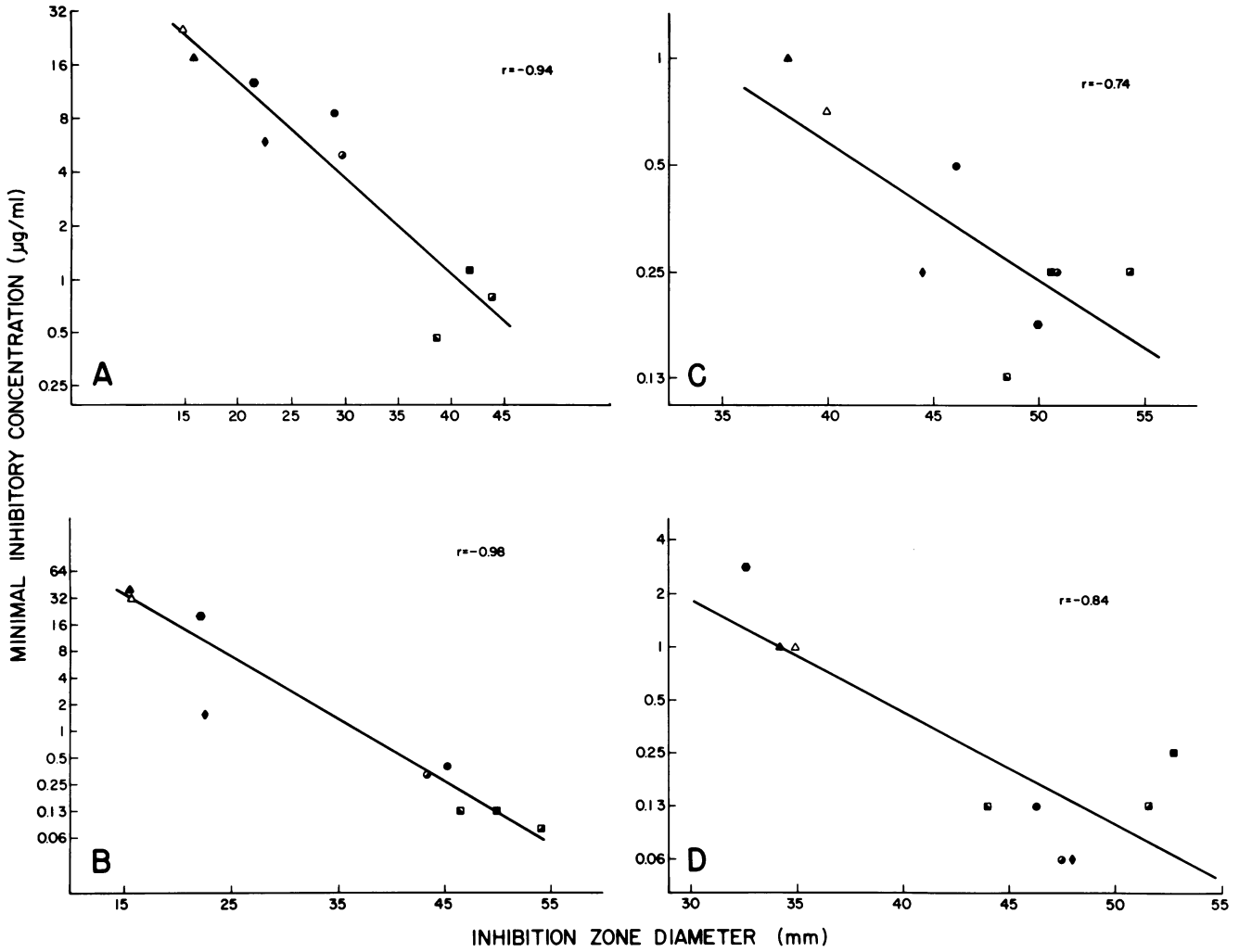

FIG. 2. Mean disk inhibition zone sizes and geometric mean MICs obtained with cephalothin (A), cefamandole (B), cefoxitin (C), and moxalactam (D) for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1.

DISCUSSION

The therapy of choice for *Legionella* pneumonia is erythromycin, with or without the addition of rifampin. Not only are all legionellae susceptible to both erythromycin and rifampin in vitro (6, 9, 11, 17, 20, 22, 23) but these antibiotics have proved efficacious in ovo (13, 16), in model infections in guinea pigs (8; A. W. Pasculle and J. N. Dowling, Program Abstr. Intersci. Conf. Antimicrob. Agents, Chemother. 22nd, Miami Beach, Fla., abstr. no. 95, 1982) and in human disease (5, 7, 12, 15, 24). Thus, most microbiology laboratories should have the capability to determine the susceptibility of *Legionella* isolates to erythromycin and rifampin. Our results indicate that disk diffusion susceptibility testing with the 15-μg erythromycin disk on BCYE agar is feasible and accurate. Since our data are based on laboratory-derived, erythromycin-resistant organisms, any clinical isolate which is found to be resistant to erythromycin by disk testing should be submitted to a reference center for agar dilution susceptibility testing. As more strains are examined by both methods, formal breakpoints for disk testing can be established. Unfortunately, the range of the MICs of the legionellae to rifampin was too small to state with assurance

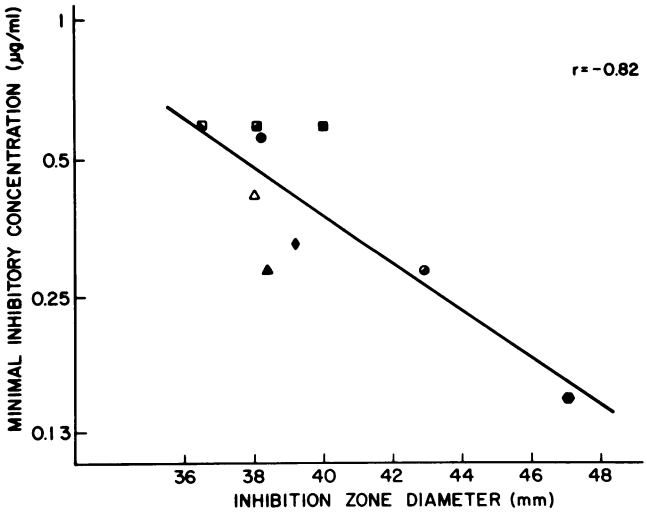

FIG. 3. Mean disk inhibition zone sizes and geometric mean MICs obtained with erythromycin for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1.

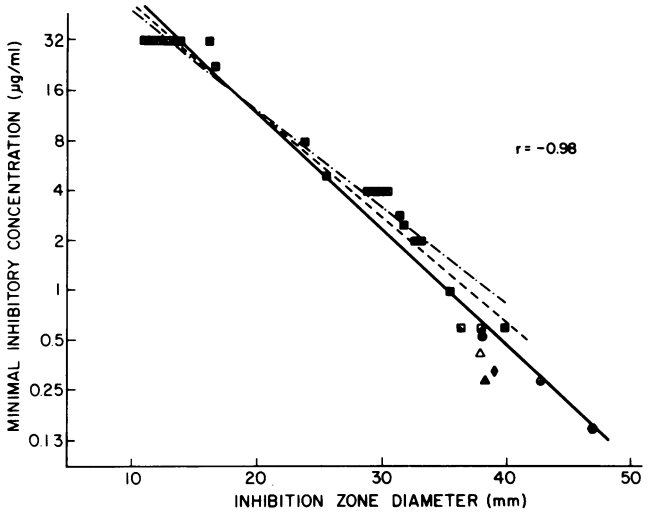

FIG. 4. Mean disk inhibition zone sizes and geometric mean MICs obtained with erythromycin for nine strains of *Legionella* and 19 erythromycin-resistant variants of *L. micdadei* EK (⊠). For symbol definitions, see legend to Fig. 1. The regression lines are shown for the erythromycin-resistant variants (– · –) ($r = -0.99$), for the resistant variants and the three naturally occurring *L. micdadei* strains (– – –) ($r = -0.98$), and for the resistant variants and the nine naturally occurring *Legionella* strains (——).

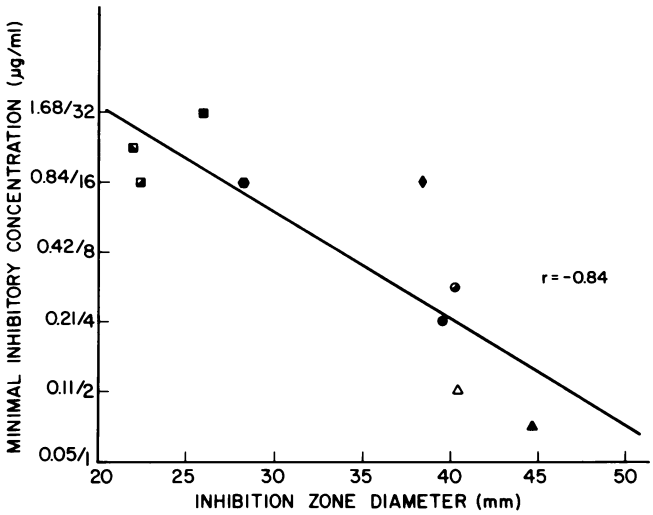

FIG. 6. Mean disk inhibition zone sites and geometric mean MICs obtained with trimethoprim-sulfamethoxazole for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1.

that disk diffusion testing is accurate. However, within the MIC range available, the regression line did appear to be linear, and highly resistant *L. micdadei* variants gave no zone around the rifampin disk. Thus, it is likely that disk diffusion testing would identify a rifampin-resistant clinical isolate, as such a strain would be expected to manifest high-level resistance.

A few patients with legionellosis have been treated with trimethoprim-sulfamethoxazole (4, 10, 12), but it has not yet been established whether this antimicrobial combination is efficacious. Disk diffusion testing appeared to be accurate for trimethoprim and could be utilized to screen legionellae for resistance to this agent. Susceptibility of the legionellae to the sulfonamides and the combination of sulfamethoxazole with trimethoprim cannot be assessed by either agar dilution or disk diffusion testing because BCYE agar contains *para*-aminobenzoic acid (17).

One of the tetracyclines has also been employed to treat leginellosis in patients who have not been able to tolerate erythromycin (12), but its efficacy has not been established. Neither disk diffusion nor agar dilution tetracycline susceptibilities can be determined for the legionellae with BCYE agar because this medium contains inhibitors of the tetracyclines (17).

Although the legionellae are sensitive in vitro to a number of additional antimicrobial agents, agents other than erythro-

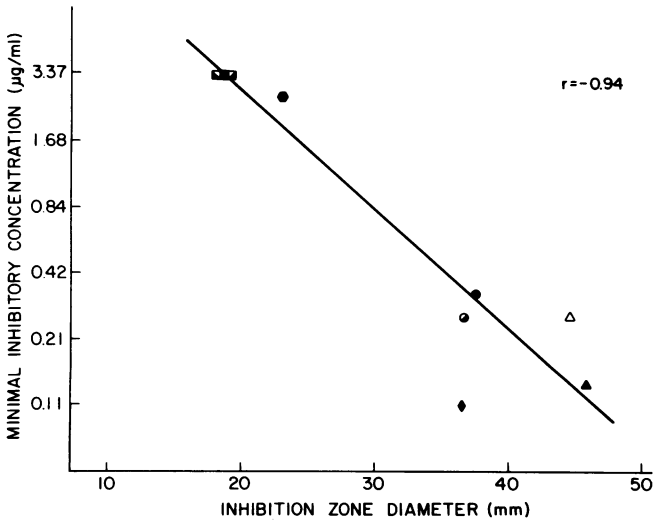

FIG. 5. Mean disk inhibition zone sizes and geometric mean MICs obtained with trimethoprim for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1.

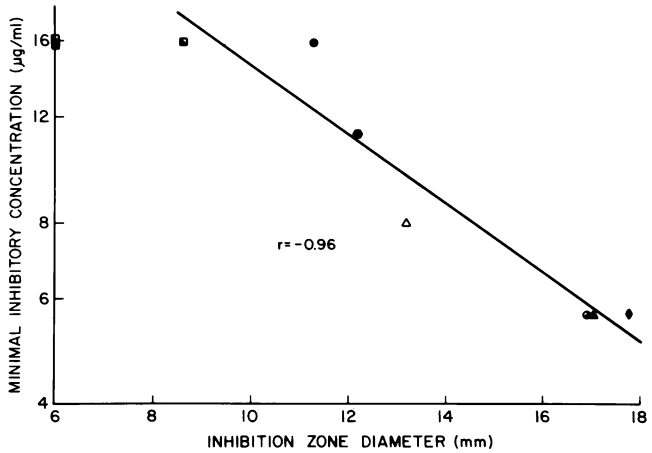

FIG. 7. Mean disk inhibition zone sizes and geometric mean MICs obtained with clindamycin for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1. The two strains for which there was no inhibitory zone were not included in the regression analysis.

mycin and rifampin, and possibly trimethoprim-sulfamethoxazole and the tetracyclines, do not appear to be effective in vivo (7, 16). Thus, there is no clinical need for susceptibility testing of the penicillins, cephalosporins, aminoglycosides, clindamycin, vancomycin, or chloramphenicol. Disk susceptibility testing with some of these agents did appear to accurately identify the legionellae to the species level. For example, testing with a number of β-lactam antibiotics could be employed for this purpose. The zone size with a carbenicillin or cefamandole disk would identify an organism as either *L. micdadei* or *L. bozemanii* as opposed to *L. pneumophila*, *L. gormanii*, or *L. dumoffii* (Fig. 1C and 2B). The cephalothin disk appears to separate *L. micdadei* from *L. bozemanii* (Fig. 2A). Testing with moxalactam identifies *L. gormanii* as opposed to *L. pneumophila* or *L. dumoffii* (Fig. 2D), and *L. pneumophila* may be separated from *L. dumoffii* by the cefoxitin disk (Fig. 2C). Alternatively, an identification scheme could start with trimethoprim disk testing, which appears to separate *L. pneumophila* and *L. micdadei* from the remaining legionellae (Fig. 5). However, since relatively few strains were examined, and because reliable methods of identification to the species level are available, disk susceptibility patterns are not presently recommended for this identification. When more data are available, disk diffusion testing might be useful for epidemiological typing or, possibly, identification of the legionellae to the species level.

It should be noted that quality control of disk susceptibility testing may not be as straightforward for the legionellae as it is for rapidly growing bacteria. The zone sizes for the control *S. aureus* and *E. coli* on BCYE agar (Table 1) were obtained by the standard Kirby-Bauer technique (3). That is, the disks were dropped on the agar immediately after the inoculum had dried, and the inhibitory zones were measured at 24 h. This contrasts to the system of delayed placement and reading at 48 h employed with the legionellae. It is possible that, for example, degradation of a particular antimicrobial agent could occur between 24 and 48 h, and

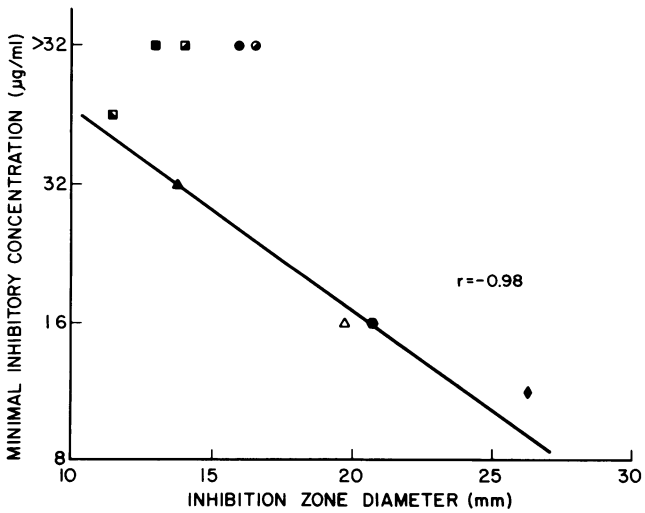

FIG. 8. Mean disk inhibition zone sizes and geometric mean MICs obtained with vancomycin for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1. The three strains with MICs greater than 32 μg/ml were not included in the regression analysis.

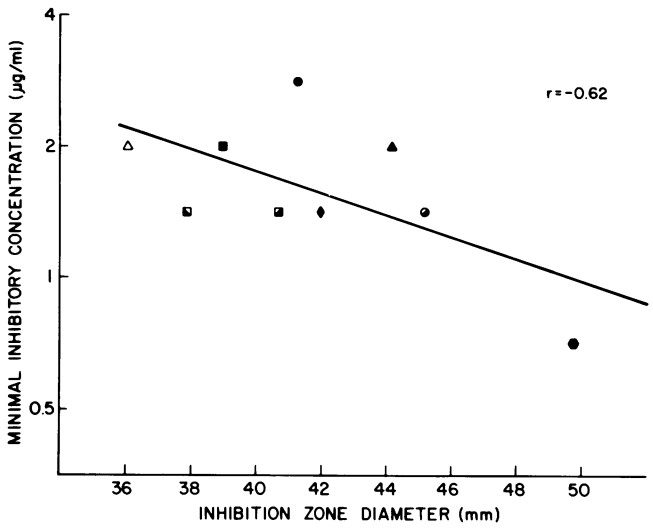

FIG. 9. Geometric mean disk inhibition zone sizes and mean MICs obtained with chloramphenicol for nine strains of *Legionella*. For symbol definitions, see legend to Fig. 1.

therefore, control of the assay with rapidly growing bacteria is not appropriate.

The ease with which we were able to produce rifampin- and erythromycin-resistant variants of *L. micdadei* deserves comment. It comes as no surprise that single-step, high-level resistance to rifampin develops with the legionellae, just as is true with other bacteria (1, 14). For this reason, rifampin should never be employed alone in the therapy of legionellosis. The development of erythromycin-resistant variants during a single passage on media containing modest concentrations of erythromycin is more ominous. This raises the possibility that erythromycin-resistant strains could be selected during erythromycin therapy of the individual patient with *Legionella* pneumonia. However, it should be noted that colonies of erythromycin-resistant variants took 1 to 2 weeks to appear on the original isolation medium. This implies that the alteration in protein synthesis required to produce resistance to erythromycin also placed the resistant variants at a growth disadvantage compared with the parent organism. On the other hand, growth of the resistant variants during the first passage on erythromycin-free medium was as rapid as that of the parent strain.

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