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Susceptibility of *Legionella* spp. to Mycinamicin I and II and Other Macrolide Antibiotics: Effects of Media Composition and Origin of Organisms

PAUL H. EDELSTEIN,[1,2]* KAREN A. PASIECZNIK,[1] VICTOR K. YASUI,[1] AND RICHARD D. MEYER[1,2]

Infectious Disease Section, Research and Medical Services, Veterans Administration Wadsworth Medical Center[1] and the Department of Medicine, University of California, Los Angeles, School of Medicine,[2] Los Angeles, California 90073

Received 3 March 1982/Accepted 4 May 1982

Thirty-three strains of *Legionella* spp., 29 of which were *L. pneumophila*, were tested for their susceptibilities to erythromycin (EM), rosaramicin, tylosin, mycinamicin I (Sch-27897), and mycinamicin II (Sch-27896). Testing was performed using an agar dilution method with two different types of media: buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEα) and filter-sterilized yeast extract medium with 0.1% α-ketoglutarate (BYEα). The minimal inhibitory concentrations (MICs) of the drugs tested relative to the MICs of erythromycin were: rosaramicin, MIC ≅ 0.2 EM MIC; tylosin, MIC ≅ 2 EM MIC; mycinamicin I, MIC ≅ 0.5 EM MIC; and mycinamicin II, MIC ≅ EM MIC. Both types of media caused equivalent partial inactivation of the macrolides which was apparently due entirely to pH effect. MICs on BCYEα were one to five times more than those observed on BYEα; this may be due to poorer growth on BYEα.

The treatment of choice for Legionnaires disease and other *Legionella* sp. infections is erythromycin (10, 12, 20). Mycinamicins are new macrolide antibiotics with antibacterial spectra similar to the spectrum of erythromycin (17). The purpose of this study was to determine whether two of the mycinamicin compounds had in vitro activity against *Legionella* sp. similar to other macrolide antibiotics (4, 7, 13, 14, 18). In addition, the effect of deletion of activated charcoal from the growth medium was determined, as it is known that charcoal yeast extract (CYE) medium inactivates many antimicrobial agents, including macrolides (4, 7, 14). This laboratory currently uses a filter-sterilized yeast extract medium without charcoal to test for pigment production of *Legionella* sp. (15). Since most recent clinical and environmental isolates of *L. pneumophila* grow readily on this medium, we felt that this medium could be used to grow *Legionella* sp. for susceptibility testing. Likewise, we felt that study of this charcoal-deleted medium could clarify whether charcoal was the interfering substance in CYE. Additional purposes were to study lower concentrations of rosaramicin than were previously tested (4) and to determine minimal inhibitory concentration (MIC) values on buffered CYE medium supplemented with α-ketoglutarate, since the addition of buffer and α-ketoglutarate to CYE medium dramatically improves the growth of *L. pneumophila* (3).

MATERIALS AND METHODS

Media. CYE medium buffered with *N*-[2-acetamido]-2-aminoethanesulfonic acid (ACES) buffer (Sigma Chemical Co.) and supplemented with 0.1% monopotassium α-ketoglutarate (BCYEα; Sigma Chemical Co.) was made as previously described (3). Buffered yeast extract medium supplemented with 0.1% monopotassium α-ketoglutarate (BYEα) was made with the same quantities of ingredients as for BCYEα except that the activated charcoal was deleted. Also, the yeast extract was filter sterilized with a 0.22-μm filter (Millipore Corp.) rather than autoclaved; it was added to the autoclaved molten cooled buffer-agar-α-ketoglutarate base at 50°C. The filter-sterilized L-cysteine and ferric pyrophosphate were then added. The media were made with 10% less water to allow for the antibiotic solution volumes. Mueller-Hinton agar (MHA) and Mueller-Hinton broth were made from dehydrated forms by the directions supplied by the manufacturers (Difco Laboratories). All three types of media (BCYEα, BYEα, and MHA) were tested simultaneously and were made on the day of use. We added 3 ml of antibiotic solution to 27 ml of cooled molten agar. This was mixed by inversion and poured into square plastic petri dishes.

Antibiotics. Standard powders of rosaramicin, tylosin, Sch-27896 (mycinamicin II), and Sch-27897 (mycinamicin I) were gifts from Schering-Plough Corp. Erythromycin gluceptate was supplied by Eli Lilly &

Co. Tylosin, Sch-27896, and Sch-27897 were first dissolved in absolute methanol and then diluted in sterile distilled water; the other agents were dissolved and diluted in sterile distilled water. Each antibiotic was tested on a separate day.

Bacterial isolates. We tested 33 *Legionella* sp. isolates; 29 of these were *L. pneumophila* strains, 8 of which were environmental isolates and 21 of which were clinical isolates. We isolated 17 of the 21 *L. pneumophila* clinical isolates and 5 of the 8 *L. pneumophila* environmental isolates at the Veterans Administration Wadsworth Medical Center (VAWMC). Two VAWMC clinical isolates were cultivated from lung specimens sent from other geographically distant institutions. One clinical and environmental isolate each of *L. pneumophila* was obtained from Atsushi Saito of the University of Nagasaki Medical School. Three clinical isolates (Philadelphia I, Togus I, and Chicago 2) and two environmental isolates (Bloomington 2 and Dallas 1E) of *L. pneumophila* were obtained from the Centers for Disease Control; all of these are type strains. Of the VAWMC *L. pneumophila* strains, only one has been specially designated (Los Angeles 1). Serogroup frequency of the *L. pneumophila* strains is as follows: serogroup 1, 19 isolates; serogroup 2, 2 isolate; serogroup 3, 1 isolates; serogroup 4, 4 isolates; serogroup 5, 2 isolates; and serogroup 6, 1 isolate. Four other species were also tested: *L. micdadei* (TATLOCK), *L. dumoffii* (TEX-KL), *L. bozemanii* (WIGA), and *L. longbeachae* (Los Angeles 24). These isolates of the other species were clinical isolates and all except one (Los Angeles 24) were obtained from the Centers for Disease Control. A total of 14 VAWMC isolates were passed on BCYEα medium between 10 and 20 times before testing. All of the isolates from Centers for Disease Control and Japan were passed multiple times on CYE or BCYEα medium. *Staphylococcus aureus* ATCC 25923 was tested at the same time as the *Legionella* strains.

All bacteria tested had been frozen at −70°C in skim milk for 1 to 24 months. They were thawed, subcultured twice on BCYEα medium, and portioned into multiple skim milk-containing freezer vials. They were then refrozen at −70°C. Before inoculation of the susceptibility testing plates, frozen samples of the organisms were thawed and passed twice (48-h incubation time) on BCYEα medium. This approach ensured that the test organisms were similar in each susceptibility run.

Inocula were prepared by harvesting growth from a 48 h plate with a sterile wooden stick into 1 ml of Mueller-Hinton broth. The turbidity of the suspension was adjusted to match that of the 0.5 MacFarland barium sulfate standard. The suspension was mixed with sterile glass beads in a Vortex blender. We have found that such a suspension of *L. pneumophila* yields 9×10^7 to 1×10^8 colony-forming units per ml; if glass beads are not used, the counts are more variable. The inoculum of *S. aureus* ATCC 25923 was prepared in a standard way (18).

Plate inoculation. A Steer's replicator was used to inoculate the plates (18). Assuming that the delivery volume is 0.001 ml, we plated $\cong 1.0 \times 10^5$ colony-forming units of the *Legionella* strains per inoculum spot. All plates were inoculated within 20 min of inoculum preparation, and duplicate plates were inoculated. Antibiotic-free plates were inoculated before and after inoculation of each set of organisms to detect contamination, antibiotic carry-over, or both.

Plate incubation and reading. Incubation was at 35°C in air with 80 to 85% relative humidity. Plates were read at 24 h for the *S. aureus* strain and 48 h for the *Legionella* strains. Less than 2 colony-forming units per spot was read as no growth, and haze was disregarded. If growth on the antibiotic-free plate was not confluent for a particular strain, results for that strain were not recorded.

Statistical analysis. All data was recorded in $\log_2$ form. Means and standard deviations were determined with these values. Comparison of means was analyzed by a two-tailed Student *t* test. The mean values recorded in Tables 1 and 2 are geometric mean values.

RESULTS

Results are shown in Table 1 for BCYEα medium. Growth on BYEα medium was poor for many strains; MIC values for this medium are thus not shown on the table. Rosaramicin MIC values were the lowest, whereas those for erythromycin, Sch-27897, and Sch-27896 were intermediate, and those for tylosin were highest.

Comparison of mean MIC values for clinical and environmental strains of *L. pneumophila* showed that in 8 of the 10 comparisons, environmental strain MIC values were higher than clinical strain values, in one case they were equivalent, and in one case the clinical strain MIC values are higher than the environmental strain values. Statistical analysis of these differences showed that in 5 of the 10 comparisons, the differences were significant ($P < 0.05$). All 10 MIC values were an average of 2 $\log_2$ dilutions lower on BYEα medium than on BCYEα medium; 9 of these comparisons were statistically significant ($P < 0.05$).

Growth of *Legionella* spp. on BYEα medium was poorer than that on BCYEα medium for some strains. Whereas all organisms reproducibly grew on BCYEα medium, only an average of 77% of strains grew on BYEα medium. The organisms which failed to grow on BYEα medium differed somewhat from run to run, but all of the *L. pneumophila* strains which grew poorly were ones which had been infrequently passed. All of the nonpneumophila *Legionella* except TEX-KL grew poorly on BYEα medium.

The effect of medium composition on antibiotic activity is shown in Table 2. There was no difference between MIC values for the *S. aureus* strain between BYEα and BCYEα media. There was a 1 to 2 $\log_2$ increase in MIC values on the yeast extract containing media over that seen on MHA. These differences were highly reproducible.

After review of these results, it was decided to determine whether the difference between the pH of MHA (7.4) and BCYEα (6.90) accounted for the difference in the *S. aureus* MIC values.

TABLE 1. Antimicrobial activity of macrolide antibiotics against *Legionella* spp.

Antibiotic	Medium	Strain group[a]	MIC (μg/ml)			
			Range	Geometric mean	MIC_{50}	MIC_{90}
Erythromycin	BCYEα	Clinical (21)	0.031–0.50	0.310	0.25	0.50
		Environ (8)	0.25–0.50	0.420	0.50	0.50
		Non-Lp (4)	0.125–0.50	0.210	0.125	0.50
Rosaramicin	BCYEα	Clinical (21)	0.015–0.125	0.067	0.063	0.125
		Environ (8)	0.063–0.125	0.088	0.063	0.125
		Non-Lp (4)	0.031–0.125	0.058	0.063	0.125
Tylosin	BCYEα	Clinical (21)	0.063–1.0	0.674	1.0	1.0
		Environ (8)	0.50–1.0	0.841	1.0	1.0
		Non-Lp (4)	0.125–2.0	0.707	0.50	2.0
Sch-27897	BCYEα	Clinical (19)[b]	0.015–0.25	0.156	0.125	0.25
		Environ (8)	0.125–0.50	0.163	0.25	0.50
		Non-Lp (4)	0.125–0.50	0.272	0.25	0.50
Sch-27896	BCYEα	Clinical (21)	0.063–1.0	0.366	0.50	0.50
		Environ (8)	0.25–1.0	0.403	0.50	0.50
		Non-Lp (4)	0.063–1.0	0.325	0.50	1.0

[a] Identification of clinical, environmental (environ), and nonpneumophila *Legionella* spp. (non-Lp) is in the text. The number of strains studied is shown in parentheses.

[b] Two strains were contaminated for this experiment, reducing the number of clinical strains observed from 21 to 19.

This was done because it was apparent that the deletion of charcoal from BCYEα did not affect the inhibition of macrolide activity. Therefore, MHA was made at pH 6.90 and 7.40, and the macrolide susceptibilities of *S. aureus* ATCC 25923 were determined on these media, and on BCYEα medium (pH 6.90). MICs on MHA at pH 6.90 were equivalent to those found on BCYEα for all the macrolides, whereas MICs on MHA at pH 7.40 were an average of 2 $\log_2$ dilutions lower.

DISCUSSION

Both of the mycinamicin drugs tested have in vitro activities against *Legionella* spp. similar to the activity of erythromycin. Rosaramicin was inhibitory at lower MIC values than was erythromycin, and tylosin, which is not used clinically, had the highest MIC values. The correlation between in vitro activity against *Legionella* spp. and clinical efficacy is not well understood, since many drugs which are inhibitory at low concentration in vitro are clinically inefficacious (10). These differences may be based on the complex interactions of respiratory tract tropism, alveolar macrophage concentration, and bacterial susceptibility to the drugs (2, 5, 8).

Before the clinical efficacy of rosaramicin and the mycinamicins can be predicted, it is important that their abilities to be concentrated within the lung and the alveolar macrophage itself be studied. Likewise, the study of their efficacy in the treatment or prevention of infection in animal models of *Legionella* infection is needed (1, 6, 10).

It is difficult to compare these results with those obtained by other investigators, because different media or methods, or both, were used. Also, with few exceptions, relatively few strains were studied by others. Regardless, our results for erythromycin MIC values are close to those found in other studies (4, 7, 13, 14, 18).

As demonstrated in another study of *L. pneumophila* in vitro susceptibility patterns, there is considerable inhomogeneity which makes the interpretation of single strain susceptibility values hazardous (4). The statistically significant differences noted in this study represent relatively small absolute differences and are probably not clinically significant. However, the differences do seem to indicate that there are consistent differences between environmental

TABLE 2. Activity of macrolide antibiotics against *S. aureus* ATCC 25923 on three different media[a]

Antibiotic	MIC (μg/ml) in following medium:		
	MHA (pH 7.4)	BYEα	BCYEα
Erythromycin	0.125	0.50	0.50
Rosaramicin	0.25	1.0	1.0
Tylosin	2.0	4.0	4.0
Sch-27896	0.25	1.0	1.0
Sch-27897	0.125	0.50	1.0

[a] Incubation was for 24 h.

and clinical isolates of *L. pneumophila*. This difference is shown but not commented on in a previously published report (13).

The deletion of activated charcoal from BCYEα medium did not change the inactivation of macrolide antibiotics. This inactivation is apparently entirely due to the pH difference between MHA and BCYEα. Since *L. pneumophila* will not grow optimally at pH 7.40, it is a moot point. It is well known that macrolide antibiotics are much more active at alkaline than at acid pH, and this finding is an example of that fact (16). McDougal and Thornsberry have made similar findings (L. K. McDougal and C. Thornsberry, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C47, p. 279).

Since growth of some of the *Legionella* spp. on BYEα medium was poor, it is unlikely that this medium can be used for their susceptibility testing. The lower MIC values demonstrated on BYEα compared with BCYEα medium most likely represent poorer growth of bacteria on BYEα medium rather than less antimicrobial agent inactivation, as evidenced by the equivalent MICs for the two media used for the *S. aureus* control strain. We do not know whether a recently described transparent medium for growth of *Legionella* spp. would behave similarly to BYEα (9). It does seem unlikely that this new medium would be useful for susceptibility testing of macrolides, since the same pH differences would be obtained. Until an optimal susceptibility testing medium for *Legionella* spp. is developed, it is mandatory that all susceptibility studies report parallel testing of the antimicrobial agent on MHA with an *S. aureus* or *E. coli* control strain and that many *Legionella* strains are studied to detect heterogeneity.

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LITERATURE CITED

1. **Baskerville, A., R. B. Fitzgeorge, M. Broster, P. Hambleton, and P. J. Dennis.** 1981. Experimental transmission of Legionnaires' disease by exposure to aerosols of *Legionella pneumophila*. Lancet ii:1389–1390.
2. **Brun, Y., F. Forey, J. P. Gamondes, A. Tebib, J. Brune, and J. Fleurette.** 1981. Levels of erythromycin in pulmonary tissue and bronchial mucus compared to those of amoxycillin. J. Antimicrob. Chemother. 8:459–466.
3. **Edelstein, P. H.** 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. J. Clin. Microbiol. 14:298–303.
4. **Edelstein, P. H., R. D. Meyer.** 1980. Susceptibility of *Legionella pneumophila* to twenty antimicrobial agents. Antimicrob. Agents Chemother. 18:403–408.
5. **Fraschini, F., P. C. Braga, V. Copponi, G. Gattei, E. Guerrasio, F. Scaglione, F. Villa, and G. Scarpazza.** 1980. Tropism of erythromycin for the respiratory system p. 659–662. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, Proceedings of the 11th International Congress of Chemotherapy, vol. 1. American Society for Microbiology, Washington, D.C.
6. **Fraser, D. W., I. K. Wachsmuth, C. Bopp, J. C. Feeley, and T. F. Tsai.** 1978. Antibiotic treatment of guinea pigs infected with agent of Legionnaires' disease. Lancet i:175–177.
7. **Hébert, G. A., C. W. Moss, L. K. McDougal, F. M. Bozeman, R. M. McKinney, and D. J. Brenner.** 1980. The rickettsia-like organisms TATLOCK (1943) and HEBA (1959): Bacteria phenotypically similar to but genetically distinct from *Legionella pneumophila* and the WIGA bacterium. Ann. Intern. Med. 92:45–52.
8. **Johnson, J. D., W. L. Hand, J. B. Francis, N. King-Thompson, and R. W. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. 95:429–439.
9. **Johnson, S. R., W. O. Schalla, K. H. Wong, and G. H. Perkins.** 1982. Simple, transparent medium for study of legionellae. J. Clin. Microbiol. 15:342–344.
10. **Kirby, B. D., K. M. Snyder, R. D. Meyer, and S. M. Finegold.** 1980. Legionnaires' disease: report of sixty-five nosocomially acquired cases and review of the literature. Medicine (Baltimore). 59:188–205.
11. **Lewis, V. J., W. L. Thacker, C. C. Shepard, and J. E. McDade.** 1978. In vivo susceptibility of the Legionnaires disease bacterium to ten antimicrobial agents. Antimicrob. Agents Chemother. 13:419–422.
12. **McKinney, R. M., R. K. Porschen, P. H. Edelstein, M. L. Bissett, P. P. Harris, S. P. Bondell, A. G. Steigerwalt, R. E. Weaver, M. E. Ein, D. S. Lindquist, R. S. Kops, and D. J. Brenner.** 1981 *Legionella longbeachae* species nova, another etiologic agent of human pneumonia. Ann. Intern. Med. 94:739–743.
13. **Orrison, L. H., W. B. Cherry, C. B. Fliermans, S. B. Dees, L. K. McDougal, and D. J. Dodd.** 1981. Characteristics of environmental isolates of *Legionella pneumophila*. Appl. Environ. Microbiol. 42:109–115.
14. **Pasculle, A. W., J. N. Dowling, R. S. Weyaut, J. M. Sniffen, L. G. Cordes, G. M. Gorman, and J. C. Feeley.** 1981. Susceptibility of Pittsburgh pneumonia agent (*Legionella micdadei*) and other newly recognized members of the genus *Legionella* to nineteen antimicrobial agents. Antimicrob. Agents Chemother. 20:793–799.
15. **Ristroph, J. D., K. W. Hedlund, and R. G. Allen.** 1980. Liquid medium for growth of *Legionella pneumophila*. J. Clin. Microbiol. 11:19–21.
16. **Sabath, L. D., D. A. Gerstein, P. Bronwen Loder, and M. Finland.** 1968. Excretion of erythromycin and its enhanced activity in urine against gram-negative bacilli with alkalinization. J. Lab. Clin. Med. 72:916–923.
17. **Satoi, S., N. Muto, M. Hayashi, T. Fujii, and M. Otani.** 1980. Mycinamicins, new macrolide antibiotics. I. Taxonomy, production, isolation, characterization, and properties. J. Antibiot. 33:364–376.
18. **Thornsberry, C., C. N. Baker, and L. A. Kirven.** 1978. In vitro activity of antimicrobial agents on Legionnaires disease bacterium. Antimicrob. Agents Chemother. 13:78–80.
19. **Washington, J. A., and V. L. Sutter.** 1980. Dilution susceptibility test: agar and macro-broth dilution procedures, p. 453–462. *In* E. H. Lennette, A. Balows, W. J. Hausler Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
20. **Wing, E. J., F. J. Schafer, and A. W. Pasculle.** 1981. Successful treatment of *Legionella micdadei* (Pittsburgh pneumonia agent) pneumonia with erythromycin. Am. J. Med. 71:836–840.