Resistance of Group A Beta-Hemolytic Streptococci to Lincomycin and Erythromycin[1]

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Ten (0.05%) of 18,628 strains of *Streptococcus pyogenes* isolated from clinical specimens in the 3 years 1968 to 1970 were resistant to lincomycin and erythromycin. All 10 strains were highly resistant to lincomycin, having minimal inhibitory concentration (MIC) values of 200 μg/ml. There were two degrees of resistance to erythromycin: four strains were highly resistant, having MIC values of 200 μg or more/ml; and six strains showed slight resistance, MIC values being 0.78 to 1.56 μg/ml. There was no known epidemiological relationship between any of the patients infected with the resistant strains, which belonged to a variety of T serotypes. A zonal pattern of resistance to lincomycin occurred in four strains, all of which were only slightly resistant to erythromycin. After incubation for 24 hr in a twofold dilution series of lincomycin in broth, the strains grew in 0.05 μg or less/ml and in 50 and 100 μg/ml, but not in intermediate concentrations. Tests in agar indicated that the bacterial population of one strain, but not of the other three, was homogeneous in respect to its ability to grow readily in low and high, but not in intermediate, concentrations. The zone phenomenon is of significance in the clinical laboratory, since unawareness of it might result in a highly resistant strain being regarded as susceptible to lincomycin in tube or plate MIC tests that do not include sufficiently high concentrations of lincomycin.

Group A beta-hemolytic streptococci (*Streptococcus pyogenes*) isolated from clinical specimens are usually highly susceptible to lincomycin and erythromycin. This is fortunate since these agents are generally regarded as the antibiotics of choice in treating infections with *S. pyogenes* in patients for whom penicillin is contraindicated. But a few instances of naturally occurring resistance have been recorded. Erythromycin-resistant strains were reported from Britain in 1959 (14), but the next reports were not made until 1968 when strains of group A beta-hemolytic streptococci resistant to both lincomycin and erythromycin were described from three countries: England (11, 15), the United States (16), and Canada (7).

We report here the incidence of strains of *S. pyogenes* resistant to erythromycin or lincomycin that were isolated from clinical specimens in Alberta during the years 1968 to 1970, including the two strains previously described (7); and we describe and draw attention to unusual features of the pattern of resistance to lincomycin shown by four of the strains.

MATERIALS AND METHODS

Isolation of streptococci. *S. pyogenes* was isolated from cotton-tipped swabs taken from patients for diagnostic or public health purposes. The swabs were inoculated on to 5% sheep blood-agar (Tryptose-blood-agar base with yeast extract; Difco) and incubated anaerobically for 18 to 24 hr. Group A beta-hemolytic streptococci were identified by precipitation with Group A antiserum (Wellcome) of bacterial extracts prepared by a modification (18) of Lancefield's acid-extraction method. The M and T type-specific antigens of certain strains were determined at the Cross-Infection Reference Laboratory, Colindale, London, England.

Antibiotic sensitivity tests. (i) For the screening disc test: all strains were tested by a disc method with discs of 2 μg of erythromycin and 2 μg of lincomycin laid on sheep blood-agar over which a colony from the primary plate had been evenly spread with a glass spreader. Incubation was at 37 C for 18 hr. Strains showing a smaller zone of inhibition than a sensitive control strain to either antibiotic were subsequently tested by tube dilution and agar plate dilution techniques with each drug.

(ii) For the tube dilution tests: a twofold dilution series of each antibiotic in Todd-Hewitt broth (BBL) was prepared in 1-ml volumes. The bacterial inoculum was 10^4 to 10^5 organisms from an appropriate dilution of an 18-hr culture in Todd-Hewitt broth. The

final concentration of antibiotic in the tubes ranged from 0.006 to 400 μg/ml or higher when necessary. The antibiotic dilutions were prepared not more than 4 hr before inoculation from sterile antibiotic powder stored at 4 C. The lincomycin powder was supplied by the Upjohn Company of Canada, and the erythromycin powder was supplied by Abbott Laboratories Limited, Montreal. The inoculated tubes were examined after incubation at 37 C for 18, 24, and 48 hr. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antibiotic in which no visible growth was observed. Optical density readings of the cultures were made with a Bausch & Lomb Spectronic 20 spectrophotometer.

(iii) For the agar plate dilution tests: a pour-plate technique was used. The bacterial inoculum was 0.1 ml of a 10⁻⁵ dilution of an 18-hr culture in Todd-Hewitt broth. The bacterial suspension and the appropriate concentration of antibiotic were added to 5% sheep blood-agar held at 45 C. This was poured on to a previously solidified layer of blood-agar base containing no blood but the same antibiotic concentration, thus forming a layered plate for easy detection of bacterial growth and hemolysis. The plates were examined after incubation at 37 C for 24 and 48 hr.

RESULTS

Incidence of antibiotic resistance. Between January 1968 and December 1970, 18,628 strains of group A beta-hemolytic streptococci isolated from clinical specimens were examined for resistance to erythromycin and lincomycin. Ten strains (0.05%) were resistant to both antibiotics; details are given in Table 1. No strains were significantly resistant to either antibiotic alone. There was a slight increase in the incidence of resistant strains during the 3-year period: in 1968, 2 (0.03%) of 6,505 strains were resistant to both drugs; in 1969, 3 (0.05%) of 5,778 strains were resistant to both drugs; and in 1970, 5 (0.08%) of 6,335 strains were resistant to both drugs.

All ten strains were highly resistant to lincomy-

cin, having MIC values of 200 μg/ml. Resistance to erythromycin was either very high or rather low; the MIC in broth of four strains was between 200 and 800 μg/ml, and of the other six strains was between 0.78 and 1.56 μg/ml. (By the technique used, the MIC of erythromycin for 50 randomly chosen sensitive strains recently isolated in our laboratory was 0.05 or less μg/ml.)

Of the strains highly resistant to both antibiotics, two (19035 and 22095) were from patients known not to have been treated with lincomycin or a macrolide antibiotic in the preceding 3 months, and one was from a patient who had received recent erythromycin therapy. Of the strains that were highly resistant to lincomycin, but only slightly to erythromycin, four were from recently treated patients, two with lincomycin and two with erythromycin.

The serotypes of the resistant strains are shown in Table 1. Ten different types are represented. The two strains of type T3/13/B3264 were isolated 8 months apart from patients living 30 miles apart. The interval between the two isolations of type M12; T12 was 2 months and the patients lived 36 miles apart.

Character of the antibiotic resistance. (i) **Erythromycin.** There was nothing noteworthy about the pattern of resistance to erythromycin in any of the 10 resistant strains when they were examined by disc, tube dilution, or agar dilution tests. The strains grew uniformly in the various concentrations of antibiotics below the MIC, except that the turbidity or colony size was sometimes reduced in the concentration immediately below the MIC, as is not uncommon in such tests.

(ii) **Lincomycin.** The four strains highly resistant to both antibiotics and two of the strains with low erythromycin and high lincomycin resistance showed no unusual features in disc, tube

TABLE 1. *Results of tube dilution susceptibility tests in Todd-Hewitt broth on 10 strains of Streptococcus pyogenes resistant to lincomycin and erythromycin[a]*

Strain designation	Source	Serotype	MIC (μg/ml) of lincomycin	MIC (μg/ml) of erythromycin	Resistance pattern with lincomycin
13234	Throat	M-; T4	200	800	Conventional
15346	Throat	M-; T8/25/Imp.19	200	200	Conventional
19035	Throat	M-; T14/Imp.19	200	400	Conventional
22095	Throat	M-; T12	200	200	Conventional
6517	Skin	M-; T3/13/B3264	200	1.56	Zonal
7509	Skin	M-; T Imp.19	200	0.78	Conventional
8374	Skin	M-; T3/13/B3264	200	0.78	Zonal
10416	Skin	M12; T12	200	0.78	Zonal
27781	Throat	M5; T5/27/44	200	1.56	Conventional
36717	Throat	M12; T12	200	1.56	Zonal

[a] The inoculum was 10⁴ to 10⁵ organisms, and the tubes were incubated at 37 C for 48 hr. MIC, minimal inhibitory concentration.

dilution, or agar dilution tests with lincomycin. But unexpected and unusual results were noted with the other four strains, all of which had low erythromycin and high lincomycin resistance. The pattern of lincomycin resistance of these strains exhibited a paradoxical zone phenomenon.

The zonal pattern was first noted in tube dilution tests with strain 6517. After incubation for 24 hr, tubes containing 0.006 to 0.05 μg of lincomycin/ml showed turbidity, the next few tubes in a series were clear, but turbidity occurred in tubes containing 50 or 100 μg/ml, or both. Initially thought to be caused by laboratory contamination, the results were found to be reproducible, and subculture from the turbid tubes showed a pure growth of the streptococcus. Similar results were obtained in tests with two different lots of lincomycin powder supplied by the manufacturer. The findings in a test with strain 6517 (type M-; T3/13/B3264) are expressed graphically in Fig. 1. Upon further incubation of the tubes, turbidity gradually appeared in the intermediate concentrations: by 48 hr it had occurred in 0.006 to 0.1 and 6.2 to 100 μg/ml; and by 72 hr in 0.006 to 0.1 and 0.79 to 100 μg/ml.

The other three strains, designated 10416 (type M12; T12), 36717 (type M12; T12), and 8374 (type M-; T3/13/B3264), behaved similarly in tube dilution tests, but the growth of strains 10416 and 36717 in the high concentrations generally did not become visible for 48 hr; an example is shown in Table 2. Determination of the MIC by reading such a test after incubation for only 24 hr would falsely indicate susceptibility of the strain, whereas the true high degree of resistance was revealed after incubation for 48 hr. Strain 8374 exhibited the zonal pattern in tube tests to a lesser extent than the other strains, slight turbidity in intermediate concentrations often being visible after incubation for 24 hr.

The occurrence of growth of these four strains in high and low, but not in intermediate, concentrations of lincomycin was confirmed in agar plate dilution tests. But these tests also indicated a fundamental difference between one strain (6517) and the other three (10416, 36717, and 8374). The appearance of some of the plates from an agar dilution test with strain 6517 is shown in Fig. 2. It is noteworthy that not only are the colonies of equal size on plates containing 0.05 and 100 μg/ml and absent on 6.3 μg/ml but also the number of colonies found on agar containing 100 μg/ml is equal to that on the antibiotic-free blood-agar. The culture is thus homogeneous in respect of its ability to grow on high and low, but not on intermediate, concentrations of lincomycin. This strain, like some of the others, forms large and small colonies; subcultures from each behaved
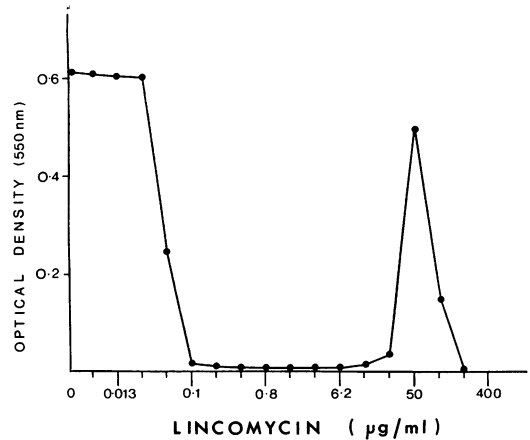

FIG. 1. Optical density of cultures of Streptococcus pyogenes strain 6517 after growth at 37 C for 24 hr in Todd-Hewitt broth containing various concentrations of lincomycin.

TABLE 2. The optical density of cultures of Streptococcus pyogenes strain 36717 after growth in Todd-Hewitt broth containing various concentrations of lincomycin[a]

Lincomycin (μg/ml)	Optical density X 100 (550 nm)		
	24 hr	48 hr	72 hr
200	0	0	0
100	0	38	57
50	0	0	51
25	0	0	0
12.5	0	0	0
6.25	0	0	0
3.13	0	0	0
1.56	0	0	0
0.78	0	0	0
0.39	0	0	0
0.2	0	0	0
0.1	0	0	0
0.05	4	10	46
0.025	62	73	74
0.013	73	74	74
0.006	68	71	73
0	68	71	73

[a] The inoculum was 2 X 10⁴ organisms, and incubation was at 37 C.

similarly in agar dilution tests with lincomycin. Tests with strains 10416, 36717, and 8374, however, gave different results, of which an example is illustrated in Fig. 3. Far fewer colonies of these strains grew on the high lincomycin concentrations than on antibiotic-free medium, indicating that only a small proportion of the bacteria in the inoculum were capable of growth on the high concentrations. The bacterial population of these
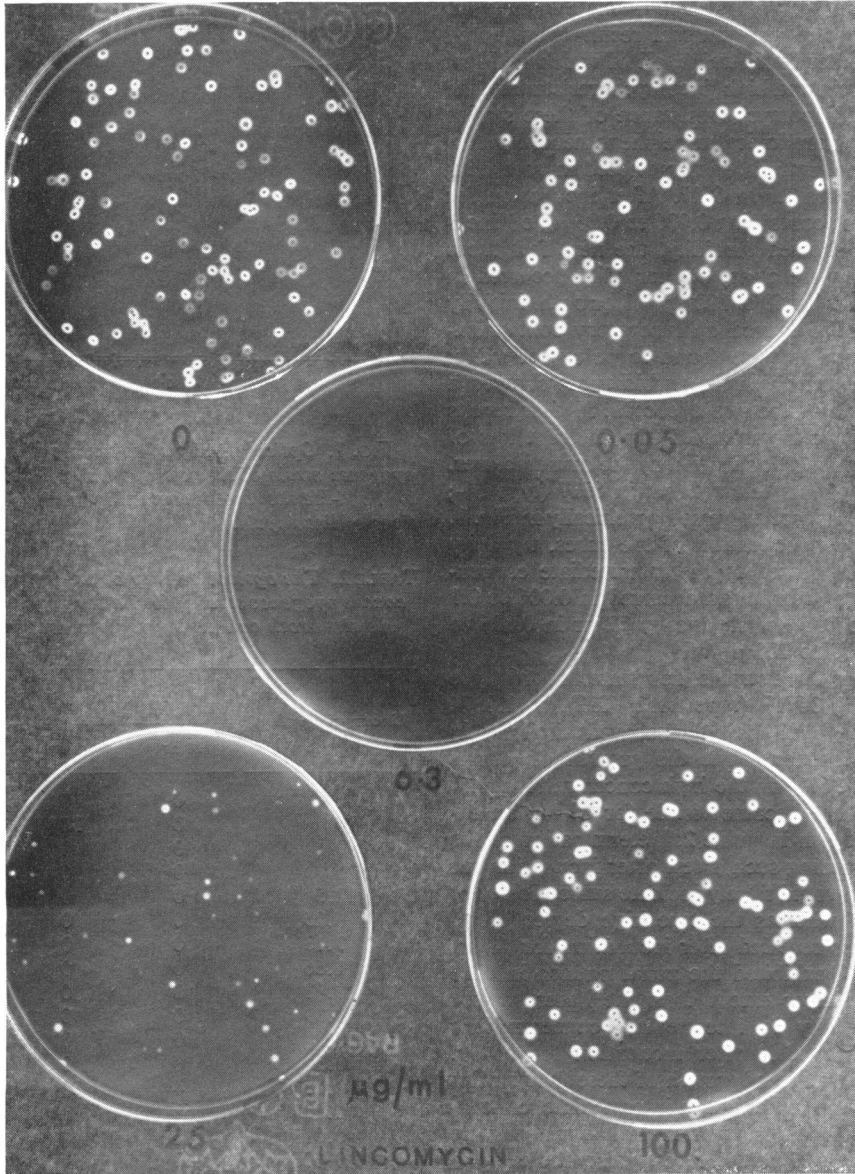
FIG. 2. *Appearance after incubation for 48 hr of some plates from an agar dilution susceptibility test to linco-mycin of Streptococcus pyogenes strain 6517.*

three strains is thus heterogeneous in respect to its ability to grow in the presence of high linco-mycin concentrations.

Organisms of strains 6517, 10416, and 36717 taken from colonies that had grown on 100 μg of lincomycin were suspended in broth and re-examined for their ability to grow on a wide range of lincomycin in pour plates. Results showed that prior growth on 100 μg lincomycin did not

alter the subsequent pattern of growth of any of the strains on lincomycin-containing media.

In disc susceptibility tests with 2-μg discs on sheep blood-agar, the homogeneous strain (6517) showed dense growth near the disc surrounded by a colony-free zone and beyond that an outer area of growth. The heterogeneous strains (10416, 36717, and 8374) showed only occasional colonies, and in some tests none, near the disc in an other-
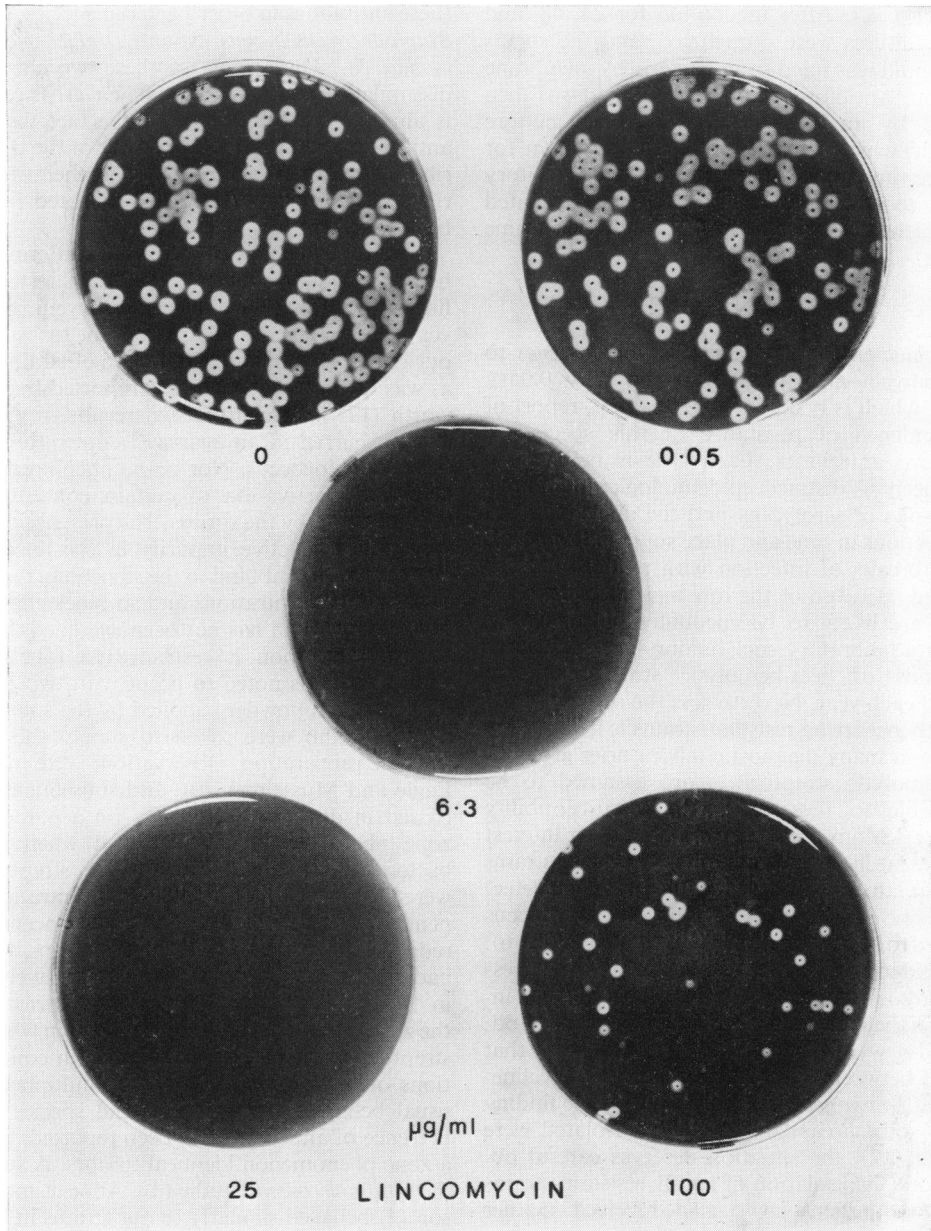
FIG. 3. *Appearance after incubation for 48 hr of some plates from an agar dilution susceptibility test to lincomycin of Streptococcus pyogenes strain 8374.*

wise wide zone free from visible growth similar in size to that of a susceptible strain. In disc tests with lincomycin, the resistance of such strains might be overlooked. (However, the zone of inhibition of these strains in a disc test with erythromycin was distinctly narrower than that of susceptible strains.)

The possibility of the zone phenomenon being the result of an impure culture was excluded by repetition of the tests a number of times with subcultures taken from single colonies. To determine whether growth of the strains in high concentrations of lincomycin was the result of destruction or inactivation of the antibiotic by the bacteria, we studied the effect on broth containing 100 µg of lincomycin per ml of the growth

of strain 6517. After incubation for 24, 48, and 72 hr, cultures were centrifuged, and the supernatant fluid was freed from bacteria by membrane filtration. Antibiotic activity of the fluid was then assayed by an agar-well method. The culture media in which the streptococci were grown for all three time periods showed identical inhibitory activity to media which had not been inoculated but which otherwise were treated in the same manner.

DISCUSSION

The incidence of resistance of *S. pyogenes* to erythromycin and lincomycin was only 0.05% in this, which is believed to be the first, report of the incidence of resistance of this species to these two antibiotics. Each case is believed to have been a distinct epidemiological incident. The results of serotyping and the distribution of the infections in time and place support this belief. No outbreaks of infection with resistant strains occurred. Because of the low incidence, resistant strains are likely to be encountered only rarely even in a laboratory such as ours in which some thousands of beta-hemolytic streptococci are isolated each year. Nevertheless the occurrence of naturally occurring resistant strains is noteworthy because in many diagnostic laboratories group A beta-hemolytic streptococci are assumed to be susceptible to these agents, and susceptibility tests are seldom or never performed. Our interest in and search for resistant streptococcal strains began in late 1967, being stimulated by the detection by one of us in 1967 of the first pneumococcus reported to be resistant to these two antibiotics (6). The incidence of resistant streptococci in our present study is low, and there was only a slight increase in their frequency during the 3-year period; but in view of the relatively short period that elapsed between the first report of a tetracycline-resistant *S. pyogenes* in 1954 (13) and the finding that in some areas 20% of strains isolated were resistant (12), the situation deserves careful observation. The isolation of highly resistant strains from two patients who had received neither lincomycin nor a macrolide antibiotic in the preceding 3 months is noteworthy. These two patients lived hundreds of miles apart, and their strains had different T antigens. Sensitivity of *S. pyogenes* to erythromycin or lincomycin should no longer be assumed by either the bacteriologist or the physician but should be confirmed by laboratory testing.

The fact that the 10 resistant strains reported here showed resistance to both erythromycin and lincomycin was not unexpected in view of reports of naturally occurring cross-resistance between these antibiotics in other bacterial species, such as *Staphylococcus aureus* (5) and *Diplococcus pneumoniae* (6). The reason for these two chemically dissimilar antibiotics often being cross-resistant is almost certainly related to the fact that both antibiotics bind to the 50S subunit of the bacterial ribosome. Competition between them for the ribosomal binding site has been noted in some bacterial species (4).

The explanation for the zonal pattern of behavior of four of our strains when tested with lincomycin, but not with erythromycin, has not yet been elicited. It was shown not to be due to our cultures containing a mixture of strains, such as was the case in an instance reported by Waterworth (17) in which unusual results, resembling ours, occurred in an agar-well susceptibility test of a staphylococcus. Nor is the phenomenon the result of inactivation of certain concentrations of lincomycin by the strains. The presence of some biologically inactive impurity in the lincomycin powder that might bind to the ribosomal receptors at certain concentrations and so block the action of the lincomycin has not been excluded. But the zone phenomenon is restricted to four of the strains and was noted to occur with two batches of lincomycin powder supplied by the sole manufacturers who were asked to supply the purest possible preparation. Observations were made by Eagle and Musselman (9), and subsequently discussed in detail by Eagle (8), on a paradoxical zone phenomenon that occurred when certain bacterial strains, including some streptococci, were exposed to increasing concentrations of penicillin. Increasing the penicillin concentration reduced the death rate of the organisms, but the bactericidal action continued progressively even in the high concentrations. This contrasts with the zone phenomenon that we report, in which the streptococci not only survived in high concentrations of lincomycin but actively multiplied. Unusual behavior of other bacterial species in the presence of antibiotics has been reported, but not a zone phenomenon identical to ours. A strain of *Serratia marcescens* studied by Annear and Hudson (2) behaved similarly to our strains in disc or cup susceptibility tests on agar when tested with colistin, but in an agar plate dilution test they reported the strain as highly resistant and made no mention of a zonal or other unusual pattern of resistance. Unusual forms of resistance of *S. aureus* to erythromycin or lincomycin have been reported by Garrod (10) and by Benner and Adams (3), but neither occurrence was similar to that reported here.

Whatever the explanation for the zonal pattern of resistance to lincomycin exhibited by four of our strains, this unusual behavior is of some

practical importance in the diagnostic microbiology laboratory. Unawareness of it might result in a highly resistant strain being regarded as susceptible to lincomycin in tube or agar plate susceptibility tests that do not include high antibiotic concentrations or are not incubated for longer than 24 hr. For example, the concentrations of lincomycin suggested as suitable for use in routine agar plate susceptibility tests in a recently published manual of clinical microbiology (1) are 10, 5, 1, and 0.1 μg/ml. With such a range of concentrations, our four resistant strains exhibiting the zone phenomenon would be regarded as susceptible. Since concentrations of lincomycin of 50 or more μg/ml are unlikely to be achieved in the blood or tissues of patients during therapy, the clinical significance of this unusual form of resistance is not known. But until it is shown to be of no consequence it should be sought, and infections caused by organisms possessing the zonal resistance pattern must be regarded as being unlikely to respond to lincomycin therapy.

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