Natural and Acquired Resistance of *Klebsiella-Aerobacter* to Cephalothin and Cephaloridine.* (30231)

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Gram negative bacteria have in recent years caused increasing numbers of hospital-acquired infections. Organisms of the *Klebsiella-Aerobacter* group have played a prominent role in this increase and treatment has frequently been difficult, partly because many strains are resistant to most of the commonly used antibiotics. Two cephalosporin C derivatives, cephalothin and cephaloridine, are new antibiotics that have shown promise in treating infection caused by Gram negative bacteria, including the *Klebsiella-Aerobacter* group. Fleming(1) observed that *Aerobacter aerogenes* strains produced cephalosporinase, an enzyme that inactivated cephalosporin C, whereas *Klebsiella* strains did not appear to produce this enzyme. Barber(2), on the other hand, found that cephalosporinase was formed by some strains of *Klebsiella* as well. These conflicting observations prompted further study of *Klebsiella-Aerobacter* organisms to determine their susceptibility to cephalothin and cephaloridine. It was found that motility of bacterial strains in this group was closely correlated with susceptibility to the 2 antibiotics. In addition, observations were made suggesting an unusual potential for development of resistance to cephalothin and cephaloridine, and this phenomenon has been explored.

Materials and methods. Bacteria. One hundred and sixty-one strains of bacteria recently isolated from patients by the microbiology laboratories of the University of Washington and King County Hospitals were classified as *Klebsiella-Aerobacter* on the basis of being Gram negative bacilli that fermented lactose, utilized citrate as their sole source of carbon, had a negative indole reaction after 48 hours incubation, and gave the expected MR-VP test results. They were further subdivided into *Klebsiella* or *Aerobacter* strains on the basis of motility according to the classification of Bergey's Manual (3), and Ewing and Edwards(4). Strains that were non-motile by both hanging-drop and semi-solid agar methods were classified as *Klebsiella,* and motile strains were considered to be *Aerobacter.*

Tube dilution minimal inhibitory concentrations (MIC's). Standard powders of cephalothin and cephaloridine were supplied by Eli Lilly and Co., Indianapolis, Ind. Each week stock solutions of the drugs were prepared in broth (tryptose phosphate, Difco) and 0.5 ml volumes were pipetted into tubes, frozen, and used within a 5-day period. An 18-hour broth culture seeded from a single colony isolate of each strain was diluted 10^{-4} in broth and 0.5 ml volumes were added to 0.5 ml of the antibiotic solutions to give final concentrations of 1000, 100, 50, 25, 20, 15, 12.5, 10, 7.5, 5, 2.5, and 1 μg/ml. After incubation of the tubes at 37°C for 18 hours, the lowest concentration of antibiotic causing complete inhibition of growth by gross inspection was considered to be the MIC.

Observations with Klebsiella *strains showing "skipped tubes."* With certain *Klebsiella* strains reading of the MIC was difficult because, following growth in 3 or 4 tubes with the lowest antibiotic concentrations, there were one or more clear tubes, and then growth in the next higher tube. Examples of this are given in Table I. This was called the "skipped tube" phenomenon and it occurred with sufficient frequency to warrant further study. With each of 13 strains that showed no skipped tubes when initially tested, the MIC determination was repeated 10 times with each antibiotic to see if the phenomenon would occur when the opportunity for its appearance was increased. All MIC values given are from tests with no skipped tubes in the series.

Where there were skipped tubes, organisms

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growing in the higher concentration tube were further tested to see how much resistance had resulted from the single exposure to antibiotic. Bacteria from this tube were diluted 10^{-4} and retested without allowing a period of growth in broth. This procedure was repeated daily to determine how quickly highly resistant strains (growth in 500 μg/ml or greater) would develop. To determine stability of this characteristic, the highly resistant strains were transferred in a high concentration of drug daily for several days, then in antibiotic-free broth for several days, and then retested. All strains were checked for purity, motility, cultural features, and biochemical characteristics every 3 to 5 days.

A second inoculum of organisms growing in the higher concentration tube after the skipped tubes was tested to determine how quickly resistant strains would appear if intermittent, rather than continuous, antibiotic exposure was used. This inoculum from the original MIC series was diluted, incubated overnight in broth, and then retested. Bacteria from the tube with the highest concentration of drug supporting growth were subcultured overnight in broth, diluted, and retested. This conventional method of testing for step-wise development of resistance was repeated 7 to 9 times in an attempt to develop appreciable resistance.

Results. Susceptibility of Klebsiella *and* Aerobacter *strains isolated from patients.* Fig. 1 shows the striking degree to which separation of *Klebsiella* from *Aerobacter* strains on the basis of motility was correlated with susceptibility to cephalothin and cephaloridine. The 130 *Klebsiella* strains were uniformly susceptible to both drugs, having MIC's of 25 μg/ml or less in all instances. Cephalothin in a concentration of 10 μg/ml or less was more active than the same amount of cephaloridine, inhibiting 71% of the *Klebsiella* strains as opposed to only 51% with cephaloridine. The 31 *Aerobacter* strains were nearly all highly resistant to both drugs. Twenty strains had MIC's of 1000 μg/ml with both antibiotics and only 2 strains were inhibited by less than 50 μg/ml.

Observations with Klebsiella *strains giving skipped tubes.* Twenty-two of the 130 *Klebsiella* strains gave skipped tubes when MIC's were initially performed with cephalothin, whereas only 10 strains showed this phenomenon with cephaloridine. Five strains having skipped tubes in the MIC tests are shown diagrammatically in Table I. When 13 organisms that failed to give skipped tubes when first examined were further tested 10 times with each drug, all strains demonstrated skipped tubes. In the 260 MIC tests performed with these 13 strains, the skipped tube phenomenon occurred 27 times. This was a frequency of approximately 10%, slightly less than the 18% noted in the screening of 130 strains. Only one *Aerobacter* strain had an MIC series with skipped tubes. It is evident that the skipped tube phenomenon is an event that occurs regularly with *Klebsiella* organisms when MIC's are performed with these two antibiotics.

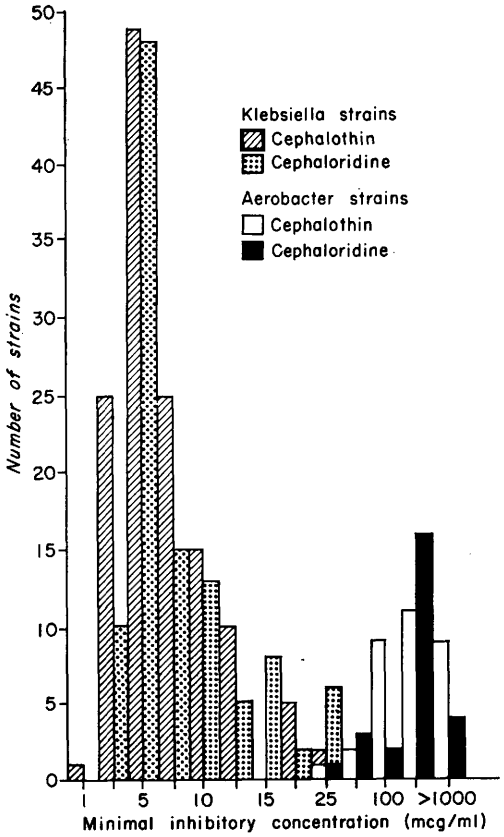

FIG. 1. *In vitro* tube dilution sensitivities of (130) *Klebsiella* and (31) *Aerobacter* strains to cephalothin and cephaloridine.

TABLE I. Representative MIC Tests Showing the Skipped Tube Phenomenon.

Strain of *Klebsiella*	Cephalothin MIC (μg/ml) 1000	100	50	25	20	15	12.5	10	7.5	5	2.5	1	Cephaloridine MIC (μg/ml) 1000	100	50	25	20	15	12.5	10	7.5	5	2.5	1
805	-	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
21046	-	-	-	+	-	+	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	+	+	+
22474	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
21479	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	+	-	+	+	+
568	-	-	-	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+

Where MIC tests showed skipped tubes, organisms growing in the higher concentration tube developed resistance rapidly when transferred daily in drug-containing media without intervening growth in antibiotic-free broth. Eleven strains of *Klebsiella* which gave skipped tubes were made to grow in 500 or 1000 μg/ml of cephalothin and cephaloridine with from 2 to 9 drug exposures. Table II demonstrates the progression of resistance. Seven strains gave visible growth in 100 μg/ml of cephaloridine after one subculture in the presence of the drug, indicating rapid one-step development of resistance. Cephalothin, on the other hand, did not allow growth at this concentration until a strain had had at least two previous exposures to the antibiotics. When tested with intermittent drug exposure (one day in drug, the next in broth), the organisms from the higher concentration tube had low MIC's that increased slightly, but never to greater than 25 μg/ml.

All strains of bacteria were tested every 3 to 5 days for purity, motility and biochemical characteristics and were unchanged. However, the development of resistance was associated with each strain giving smaller sized colonies on blood agar and MacConkey agar plates as compared to the parent strain. Small colonies occurred only after the strain was able to grow in 100 μg/ml or more of the drug. Each small colony strain remained non-motile, fermented lactose, utilized citrate as the sole source of carbon, and gave a negative indole reaction. Once able to grow in 500 to 1000 μg/ml of either antibiotic, each strain retained this high degree of resistance despite as many as 9 passages in antibiotic-free broth and the small colony feature was unchanged.

Discussion. These studies show a distinct separation between *Klebsiella* and *Aerobacter* strains on the basis of susceptibility to the cephalosporin antibiotics that is of practical value as well as of taxonomic interest. Preliminary identification of an organism as a member of the *Klebsiella-Aerobacter* group can be promptly followed by a motility test, with a strong likelihood of susceptibility to cephalothin and cephaloridine if it is non-

TABLE II. Development of Resistance in *Klebsiella* Strains Transferred Daily in Advancing Increments of Two Cephalosporin Antibiotics. Concentrations in parentheses are cephaloridine (40602) values, the others are cephalothin (Keflin).

Strain of *Klebsiella*	Drug	MIC without "skipped tubes"	Tube with growth after "skipped tubes"	No. of drug exposures — Highest concentration of drug allowing growth with each subculture (μg/ml)										
				1	2	3	4	5	6	7	8	9	10	11
805	Keflin	7.5	12.5	15	25	100	100	100	250	250	250	500	500	1000
	40602	(5)	—	(50)	(50)	(100)	(100)	(100)	(250)	(250)	(250)	(500)	(500)	(500)
568	Keflin	10	25	50	100	100	100	250	250	500	500	500	500	500
	40602	(15)	—	(100)	(100)	(100)	(100)	(100)	(250)	(500)	(500)	(500)	(500)	(500)
684	Keflin	7.5	20	25	50	50	100	250	250	500	500	500	500	500
	40602	(5)	—	(10)	(10)	(50)	(100)	(100)	(100)	(250)	(500)	(500)	(500)	(500)
639	Keflin	2.5	12.5	15	100	100	100	100	250	250	500	1000	1000	1000
	40602	(7.5)	—	(100)	(100)	(100)	(100)	(500)	(500)	(500)	(500)	(1000)	(1000)	(1000)
21046	Keflin	12.5	—	50	50	100	100	100	100	250	500	1000	1000	1000
	40602	(15)	(50)	(100)	(100)	(100)	(250)	(500)	(500)	(500)	(1000)	(1000)	(1000)	(1000)
21159	Keflin	5	12.5	25	100	100	100	100	500	1000	1000	1000	1000	1000
	40602	(20)	—	(100)	(100)	(100)	(250)	(250)	(250)	(500)	(1000)	(1000)	(1000)	(1000)
21498	Keflin	10	25	25	50	50	100	100	250	250	500	500	500	500
	40602	(20)	—	(100)	(100)	(250)	(500)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)
21479	Keflin	15	25	50	50	100	100	250	250	250	1000	1000	1000	1000
	40602	(20)	—	(25)	(50)	(100)	(100)	(250)	(250)	(250)	(500)	(1000)	(1000)	(1000)
19	Keflin	10	20	50	50	50	100	100	250	250	500	500	500	500
	40602	(12.5)	—	(50)	(50)	(100)	(250)	(500)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)
23001	Keflin	12.5	25	50	50	100	100	250	250	1000	1000	1000	1000	1000
	40602	(25)	—	(100)	(500)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)	(1000)
22474	Keflin	5	—	50	50	250	250	500	500	500	500	500	500	500
	40602	(10)	(50)	(100)	(100)	(500)	(500)	(500)	(500)	(500)	(500)	(500)	(500)	(500)

motile, and the probability that it will be resistant to these antibiotics if it is motile. Our study of this phenomenon was prompted by Fleming's observation(1) that the motile strains (*Aerobacter*) produced cephalosporinase whereas the non-motile strains (*Klebsiella*) did not. Using a different method, Barber(2) demonstrated that some *Klebsiella* strains were cephalosporinase producers, and at present it cannot be concluded that resistance to these antibiotics is necessarily based on production of this enzyme. Interestingly, our *Aerobacter* strains were all susceptible to tetracycline, chloramphenicol, streptomycin, kanamycin, furadantin, and sulfonamides when tested by the single disc method(5), whereas over a third of the *Klebsiella* strains were resistant to most of these drugs. This latter point enhances the importance of susceptibility of *Klebsiella* strains to the cephalosporin antibiotics.

The frequent occurrence of skipped tubes when MIC's were performed with *Klebsiella* strongly suggests that resistance to the cephalosporin antibiotics may readily occur during clinical therapy. Previously, the skipped tube phenomenon occurred in our laboratory with consistency only when staphylococci were tested with novobiocin and fucidin, and widespread clinical use of these two antibiotics led to the rapid appearance of resistant strains. Clinical experience with cephalothin and cephaloridine is still quite limited, but Anderson and Petersdorf(6), and also Stewart(7), observed resistant *Klebsiella-Aerobacter* strains following therapy. The possibility that these were superinfections with resistant *Aerobacter* strains was not excluded. Weinstein(8) found that laboratory resistance could be induced much more readily with Gram negative than with Gram positive bacteria and a stepwise increase in resistance of *Klebsiella* strains to cephalothin was observed by Godzeski(9). The skipped tube phenomenon has not been reported by others, but this may be due in part to the fact that the increments between the tubes in our MIC tests were less than 2-fold. The exact mechanism has not been worked out, but the rapidity of the appearance of a large degree of resistance when *Klebsiella* are transferred in the presence of drug suggests a high mutation rate. Cephalosporinase may play a role, but the selection of inherently drug-resistant mutants seems a more likely explanation. Once a high degree of resistance was attained by our *Klebsiella* strains, the colonies were smaller than those of the original culture. This is possibly a point against the clinical significance of the phenomenon since with other bacteria, notably some staphylococci, small colony variants have been thought to be considerably reduced in their virulence. It is evident that careful clinical studies of patients treated with these agents in whom *Klebsiella* strains are not promptly eliminated will be necessary to determine whether resistance to the cephalosporin antibiotics will occur with unusual frequency. Such studies are under way.

Summary. Of 161 recently isolated *Klebsiella-Aerobacter* strains, nearly all motile strains (*Aerobacter*) were highly resistant to cephalothin and cephaloridine, but susceptible to tetracycline, chloramphenicol, streptomycin, kanamycin, furadantin, and the sulfonamides. In contrast, all non-motile strains (*Klebsiella*) were susceptible to both cephalothin and cephaloridine, more so to the former, but were frequently resistant to several of the other 6 drugs. MIC tests with *Klebsiella* strains frequently demonstrated inconsistent end points with one or more clear tubes occurring between tubes showing growth. This "skipped tube" phenomenon suggested rapidly acquired resistance to these cephalosporin derivatives. The rapid *in vitro* development of a high degree of resistance by serial transfer of the organisms in these antibiotics further suggests that clinically resistant infections by these organisms may occur with unusual frequency.

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Atherogenesis and Thrombogenesis in the Rat.* (30232)

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The relationship between atherosclerosis and thrombosis has been much investigated in recent years. Generally thrombosis has been regarded as a phenomenon secondary to atherosclerosis. Other possibilities have also been considered; namely, a) that atheromatous lesions may originate as mural thrombi and, b) that the 2 processes are independent of each other. The latter hypothesis in particular has been supported by recent publications claiming that certain diets high in saturated fats induce intravascular thrombosis in the rat with little structural changes in the vascular endothelium(1). Another report claimed the independent production of atherosclerosis and thrombosis by diets differing in fat composition(2). In view of such reports, our experience with similar diets in induction of cardiovascular lesions in the rat is presented here.

Materials and methods. Male Wistar albino rats weighing about 300 g were used. The diets given were the variations of the original diet used by Fillios and associates(3) and later modified by Thomas and Hartroft (1). The composition of these diets is given in Table I. The composition of the diet in Group I is similar to the diet reported to cause intravascular thrombosis and visceral infarction but little structural changes(1,2). In Groups II and III corn oil was substituted for 40% butter at a level of 40% and 8% respectively. These diets are similar to the ones reported to cause atherosclerosis (2,3).

TABLE I. Composition of Diets in the Various Groups in Percentage by Weight.

Ingredients	Group I	Group II	Group III
Cholesterol	5.0	5.0	5.0
Propylthiouracil	.3	.3	.3
Sodium cholate	2.0	2.0	2.0
Casein	20.0	20.0	20.0
Salt mixture	4.0	4.0	4.0
Vitamin mixture	2.0	2.0	2.0
Alphacel	6.0	6.0	6.0
Choline chloride	.2	.2	.2
Sugar	20.5	20.5	52.5
Butter	40.0	—	—
Corn oil	—	40.0	8.0

Pathology. Complete autopsies were done on all animals as soon as possible after death. Details of pathological methods employed have been published(4). Since few lesions were found in those animals that were maintained on the diet for less than 100 days, the analysis of the pathological results is based on the findings in animals that came to the autopsy after 100 days or more of the experimental period. Each animal was housed in an individual cage and fed *ad lib.*

Results and discussion. In the early weeks of the experiment there was loss of weight and a high incidence of mortality especially in the group receiving 40% butter. During this early period the animals that came to autopsy did not show significant intimal disease but rather showed the presence often of thrombus-like masses in cardiac chambers

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