GENETIC STUDIES ON THE DEVELOPMENT OF STREPTOMYCIN RESISTANCE IN PASTEURELLA PESTIS[1]

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Meyer and Quan (1949) have demonstrated amply the therapeutic efficacy of streptomycin in experimental bubonic and pneumonic plague. Since streptomycin appears to be unique among the antibiotics in that high levels of resistance may occur in bacteria at a single mutational step (Bryson and Demerec, 1950), the occurrence of strains of *Pasteurella pestis* so resistant to this antibiotic as to be refractory to therapy would represent a major problem in the treatment of plague, especially in endemic areas. Consequently, a study of the factors underlying the efficacy of streptomycin therapy in plague as well as the probability of the occurrence of streptomycin resistance during the course of such therapy was undertaken.

MATERIALS

One virulent strain (139-L) and two avirulent strains (A-1122 smooth, and A-1122NS non-smooth) of *Pasteurella pestis* were used in this study. Liquid cultures were grown in tryptose broth (Difco), and platings were made on the surface of tryptose agar plates since pour plates were found to be unsatisfactory. One unit of streptomycin sulfate (Pfizer) was equivalent to one microgram of streptomycin base.

Mice, Namru strain, (Garber and Hauth, 1950) and guinea pigs were used in virulence studies. *P. pestis*, strain 139-L, is highly virulent (LD_{50} = 3 to 9 cells) when inoculated subcutaneously into these hosts.

EXPERIMENTAL RESULTS

The effect of streptomycin on large populations of *P. pestis*, strain A-1122, was determined by inoculating plates and broth containing different concentrations of the antibiotic with 2×10^9 viable cells. Numerous colonies survived concentrations of 2, 4, and 8 units per ml in agar; a total of ten colonies survived 16, 32, . . . 1,024 units per ml. Colonies surviving 2, 4, 8, 16, and 128 units per ml were tested for resistance to 25 units per ml or dependence (table 1). Colonies resistant to 25 units per ml were also resistant to 1,000 units per ml and were considered fully resistant. Both sensitive and fully resistant colonies were found among survivors from plates with 2 to 16 units per ml; only fully resistant colonies survived 128 units per ml.

TABLE 1

*The frequencies of resistant colonies in cultures of Pasteurella pestis, strain A-1122, plated in triplicate on different concentrations of streptomycin**

STREPTOMYCIN CONCENTRATION (UNITS/ML)	NO. OF COLONIES TESTED	NO. OF COLONIES RESISTANT TO 25 UNITS/ML	NO. OF DEPENDENT COLONIES	PERCENTAGE RESISTANT COLONIES
2	27	2	0	7.5
4	14	2	0	14.3
8	20	0	0	0
16	8	0	2	0
128	2	2	0	100.0

* Inoculum—2×10^9 viable cells.

A series of liquid cultures containing 2×10^9 viable cells of strain A-1122 and graded concentrations of streptomycin was incubated for 12 days (table 2). It was possible to sterilize cultures with as low as 2 units per ml. All cultures surviving 32 to 1,024 units per ml were fully resistant. The rate of sterilization in broth was determined for strains A-1122 and A-1122NS. Flasks containing 2×10^9 viable cells and 25 units per ml were sterile within 4 to 5 hours.

The effect of streptomycin was determined

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TABLE 2

The effect of streptomycin on liquid cultures of Pasteurella pestis, strain A-1122, after 12 days' incubation

STREPTOMYCIN CONCENTRATION (UNITS/ML)	VIABLE/TOTAL*	NO. OF CULTURES RESISTANT TO 1,000 UNITS/ML
2	5/9	1/5
4	2/9	1/2
8	1/9	0/1
16	0/9	0/0
32	2/9	2/2
64	1/9	1/1
128	2/9	2/2
256	3/9	3/3
512	1/9	1/1
1024	0/9	0/0

* Inoculum—2×10^9 viable cells/culture.

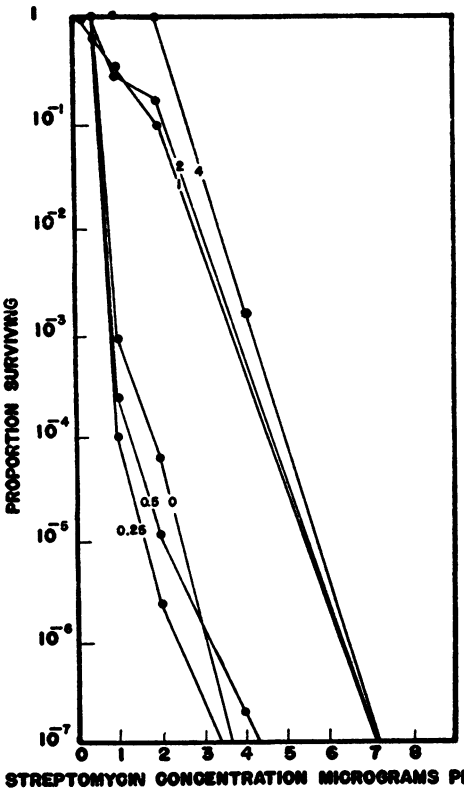

Figure 1. Survival curves for the avirulent strain A-1122, *Pasteurella pestis*. The number in each curve indicates the streptomycin concentration in units per ml of medium from which the colony was isolated.

quantitatively by plating known numbers of cells on the surface of plates containing graded concentrations of the antibiotic. The proportion of surviving bacteria was established by comparing the number of colonies appearing on incubation with the number of bacteria plated. Survival curves were obtained for strains A-1122 and 139-L and for cultures derived from single

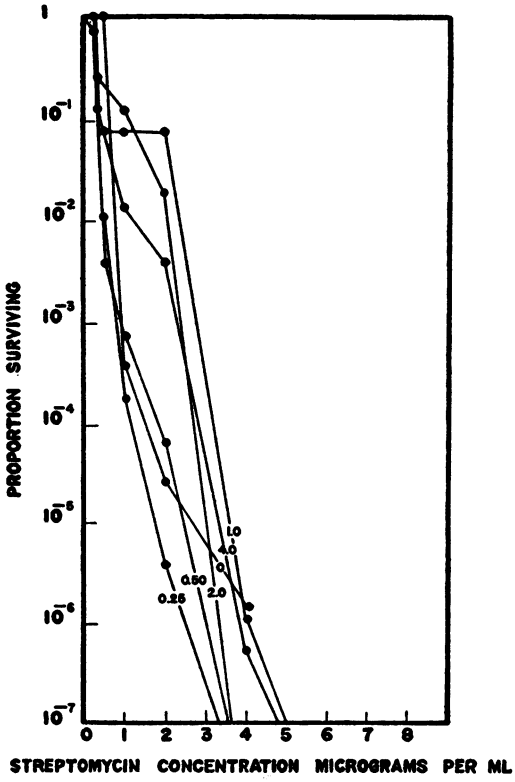

Figure 2. Survival curves for the virulent strain 139-L, *Pasteurella pestis*. The number in each curve indicates the streptomycin concentration in units per ml of medium from which the colony was isolated.

colonies surviving 0.25, 0.5, 1, 2, 4, and 8 units of streptomycin per ml in each strain (figures 1 and 2). Both strains 139-L and A-1122 were extremely sensitive to low concentrations of streptomycin. One unit of streptomycin per ml was sufficient to produce a thousandfold decrease in viable count. Plates containing 1, 2, and 4 units per ml required 3 to 5 days of incubation compared with 2 days required for either the control cultures or plates containing 0.25 and 0.5 units

TABLE 3

Mutation rates from streptomycin sensitivity to streptomycin resistance in Pasteurella pestis

STRAIN	C	VOLUME ML	$N_1 \times 10^9$	P_0	r	$a_1 \times 10^{-11}$	$a_2 \times 10^{-11}$
A-1122	100	1	1.4	0.96	0.04	2.0	1.6
	100	1	2.2	0.98	0.04	0.6	1.0
	100	1	4.2	0.97	0.03	0.5	0.5
	100	2	5.7	0.96	—	0.5	—
Average mutation rate						0.9	1.0
A-1122NS	100	1	1.0	0.97	0.07	2.1	3.2
	100	1	2.0	0.98	0.07	0.7	1.6
	100	1	2.2	0.99	0.03	3.1	0.9
Average mutation rate						2.0	1.9
139-L*	100	1	10.0	1.00	—	—	—
	100	1	10.0	0.99	0.01	—	—
	100	1	13.0	0.99	0.01	—	—

C, number of cultures; N_1, average number of viable bacteria in the cultures; P_0, proportion of cultures lacking any mutants; r, average number of mutants per culture; a_1 and a_2, estimates of mutation rate using formulae 1 and 2 (Newcombe, 1948).

* Medium fortified with 0.8 per cent yeast extract (Difco).

per ml. The 2 families of curves for strain A-1122 (figure 1) and the single family of curves for strain 139-L (figure 2) may not reflect a strain difference since each curve was based on a single colony.

Twenty resistant colonies from strain A-1122 were subcultured serially in the absence of streptomycin both on solid and in liquid media. Isolated colonies from a previous culture served as inoculum for the solid medium; a loopful from a previous culture was used for inoculating the liquid media. No change in resistance to 1,000 units per ml was noted after 10 such subcultures.

Replicate cultures were grown from small inocula (1 to 3 $\times 10^2$ viable cells), incubated for 48 hours at 22 C on a shaker, and the entire contents were spread on tryptose agar plates containing 25 units of streptomycin per ml. Colony counts were made and each colony was tested for complete resistance or dependence. In one experiment with strain A-1122, sufficient streptomycin to yield 25 units per ml was added to the cultures which then were incubated and tested for viability. Mutation rates to streptomycin resistance in strains A-1122 and A-1122NS, estimated by the 2 usual methods (Luria and Delbrück, 1943), were approximately 10^{-11} per bacterium per division cycle and in strain 139-L, approximately 10^{-12} per bacterium per division cycle (table 3). The mutation rate estimated by each method for each strain was approximately equal as was found also in *Escherichia coli* (Newcombe and Hawirko, 1949). Of the 31 colonies surviving 25 units per ml in all experiments, only 1 colony was streptomycin dependent.

TABLE 4

Virulence and streptomycin resistance in Pasteurella pestis, strain 139-L, as determined by subcutaneous inoculation in mice and guinea pigs

RESISTANT CLONE	INOCULUM (VIABLE CELLS)	MICE DEAD/TOTAL	INOCULUM (VIABLE CELLS)	GUINEA PIGS DEAD/TOTAL
1	240	9/10	6.1×10^5	1/2
2	260	8/10	6.5×10^5	2/2
3	40	0/10	1.0×10^4	0/2
4	58	0/10	2.9×10^4	0/2
5	114	0/10	1.5×10^4	0/2
6	48	0/10	1.2×10^4	0/2
7	200	8/10	0.5×10^4	2/2

Fifty plates of tryptose agar containing 25 units of streptomycin per ml of medium were each seeded with 1 $\times 10^8$ viable cells, each plate

receiving an 0.1 ml aliquot from a 48 hour culture of the virulent strain 139-L. Seven fully resistant colonies were obtained. These colonies then were tested for virulence by inoculating known numbers of viable cells subcutaneously into mice and guinea pigs. Of the 7 colonies, 3 were virulent in both hosts (table 4). Cultures from autopsied animals were fully resistant.

The therapeutic value of streptomycin in experimental plague was evaluated in terms of subeffective and effective treatment. Therapy was initiated 48 hours after a subcutaneous inoculation of approximately 1×10^3 viable cells of either a streptomycin sensitive or resistant strain into mice. In the subeffective treatment, 2,000 units of streptomycin were administered twice subcutaneously within 7 hours to 40 mice of which half had received the sensitive strain and half the resistant strain. A similar number of mice inoculated with each strain were held as a control. Although all mice given subeffective therapy died within 9 days after inoculation, a marked difference in survival up to 6 days was noted between the two groups. Whereas 100 per cent of the mice inoculated with the resistant strain had died, only 25 per cent of the mice inoculated with the sensitive strain had succumbed. In the effective therapy, 2,000 units were administered subcutaneously twice within 7 hours for 4 successive days to 20 mice of which half had been inoculated with the sensitive strain and half with the resistant strain. An equal number of mice inoculated with each strain were held as a control. Although this treatment was effective for mice inoculated with the sensitive strain, since only 1 mouse died within 21 days, none of the mice inoculated with the resistant strain survived. All the mice held as controls succumbed. It should be noted that mice surviving up to 21 days usually do not die subsequently of plague.

DISCUSSION

Ample evidence has been reported in the literature for the mutational origin of streptomycin resistance. Klein and Kimmelman (1946) demonstrated the presence of resistant cells of *Shigella dysenteriae* independent of the action of streptomycin. Newcombe and Hawirko (1949) compared the numbers of resistant cells in individual test cultures with the numbers expected according to the mutation hypothesis in *E. coli* and found good agreement. Finally, Hsie and Bryson (1950) considered the ability of streptomycin resistant stocks of *Mycobacterium ranae* to maintain their resistance in the absence of the antibiotic as evidence for their mutational origin.

A mutation rate may be considered as an estimate of the probable occurrence of a mutant in a population of proliferating cells. If the mutation rate is low, large populations are required to detect the mutant. The relatively low mutation rate and high degree of sensitivity of *P. pestis* to streptomycin are responsible for the sterilization of cultures containing 10^9 viable cells with concentrations as low as 2 units per ml. This situation permits a considerable range of internal (host) levels of streptomycin concentrations to be effective in the therapeutic treatment of plague.

Four of the 7 fully resistant colonies recovered from 5×10^9 viable cells of strain 139-L exposed to 25 units of streptomycin per ml of medium were avirulent. These avirulent colonies may have originated either from resistant, avirulent cells or from resistant cells that had lost their virulence as a consequence of the mutation to streptomycin resistance. Since avirulence in *P. pestis* does not lead to streptomycin resistance, 2 mutations would be required to yield a resistant, avirulent cell. This interpretation must consider the relatively low mutation rate, 10^{-12}, for streptomycin resistance and the relatively high virulence (LD_{50} = 3 to 9) of strain 139-L. It appears more likely that a mutation resulting in streptomycin resistance may lead also to avirulence. A mutation to streptomycin resistance in *E. coli* may result also in an alteration in other characters, such as growth rate, duration of lag period, and nutritional requirements (Demerec, 1950).

Although resistant, virulent strains of *P. pestis* are refractory to streptomycin, the occurrence of such strains in the therapeutic treatment of plague is not considered a potential hazard due to a combination of 3 factors: high sensitivity to relatively low concentrations of streptomycin, a relatively low mutation rate from sensitivity to resistance, and the lack of absolute correlation between virulence and resistance in resistant mutants from virulent strains. Consequently, the few virulent, resistant cells that may occur after streptomycin therapy would probably not survive the defensive mechanism of the host.

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SUMMARY

Pasteurella pestis is very sensitive to streptomycin *in vitro* and *in vivo*. A concentration of 1 unit per ml of medium was sufficient to produce a thousandfold reduction in the number of viable cells on solid medium, and a concentration of 2 units per ml of media, to sterilize liquid cultures. The mutation rate from streptomycin sensitivity to resistance was relatively low: about 10^{-11} in avirulent strains and about 10^{-12} in a virulent strain 139-L. Streptomycin resistance and virulence were not correlated absolutely in resistant mutants derived from a virulent strain. Streptomycin therapy effective for mice inoculated with a sensitive, virulent strain was ineffective for mice inoculated with a resistant, virulent strain.

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