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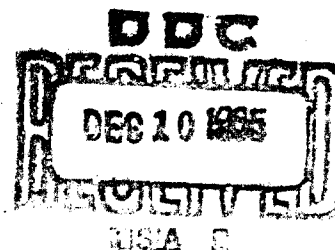
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TECHNICAL MANUSCRIPT 254

PLAGUE: GROWTH TEMPERATURE,
VIRULENCE, AND THE GRADED RESPONSE

Albert J. Rosenwald
Ralph E. Lincoln

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UNITED STATES ARMY
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**U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland**

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**PLAGUE: GROWTH TEMPERATURE,
VIRULENCE, AND THE GRADED RESPONSE**

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**Process Development Division
DIRECTORATE OF DEVELOPMENT**

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October 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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ABSTRACT

The virulence of Pasteurella pestis was compared by the graded and quantal response methods. Both tests reflected the difference in virulence of cultures grown at three temperatures. Cultures grown at lower temperatures gave the most variable response in virulence tests and cultures grown at higher temperatures were more virulent. Results from the graded response test were obtained more quickly and more economically than those from the quantal response test.

I. INTRODUCTION

The virulence of Pasteurella pestis is measured most often by the quantal response (LD_{50}); however, the innate variability in this method together with the variable response elicited by plague organisms, particularly those grown at 26 to 28 C, often results in data with wide confidence limits. The graded response (median time to death) is another method of determining virulence, a method that does not appear to have been applied to the plague organism. A comparison of the quantal and graded response methods has been reported previously.¹⁻⁴ This report describes the use of the graded and quantal responses in comparing the virulence of plague cultures grown at different temperatures.

II. MATERIALS AND METHODS

A. BACTERIAL STRAIN

Pasteurella pestis strain L-37, obtained from the Microbiological Research Establishment, Porton, England, was used in these studies. It is virulent and, in its present unselected state, carries in it a low level of avirulent cell types as determined by colony differentiation on hemin agar and on magnesium oxalate agar.

B. CULTURAL CONDITIONS

The components of the liquid growth medium were N-Z Amine Type A, 3.0% (Sheffield Chemical, Norwich, N. Y.); yeast extract, 1.0% (Difco); K_2HPO_4 , 0.4%; in tap water at pH 7.1 ± 0.1 . Two per cent galactose (final concentration) was added aseptically after sterilization. This medium was used at a volume of 25 ml in a 250-ml Erlenmeyer flask to which 1 ml of liquid culture was added as inoculum. Liquid cultures were shaken for 24 hours on a reciprocating shaker (100 three-inch strokes per min) at the desired temperature of growth.

Viable cells were counted by surface plating suitable dilutions of the bacteria on Difco blood agar base medium (BAB) supplemented with 0.1% glucose and 0.04% sodium sulfite. The magnesium oxalate agar (MOM) was described by Higuchi and Smith.⁵ These two media were incubated, after inoculation, for 40 to 48 hours, the BAB at 26 C and the MOM at 37 C. The inoculated hemin agar plates⁶ were incubated for 96 hours at 26 C.

The diluent used for all purposes was composed of N-Z Amine Type A, 0.5%, and NaCl, 0.5%, in distilled water at pH 7.2.

C. VIRULENCE TESTS

The quantal response was determined by intraperitoneal injection of Swiss-Webster mice (16 to 20 grams) with 0.2 ml of suitable dilutions of the culture. Six doses with 10 mice per dose were used in each titration and, after 10 days, the LD_{50} was calculated according to the method of Litchfield and Wilcoxon.⁷

The graded response was determined by intraperitoneal challenge of part of the same group of mice with 0.5 ml of a dilution of the culture. Doses spaced one log apart, with 10 mice per dose, were used in each titration. As a toxin control, culture supernatant fluid, sterilized by filtration, was diluted to the least dilution used in the titration and used to challenge mice. The mice were checked hourly and then at more frequent intervals when deaths began to occur. The elapsed period between time of challenge and the average of the time to death of the fifth and sixth animals was taken as the median time to death (MTD).

III. RESULTS AND DISCUSSION

Typical data in the comparison of the graded and quantal response of mice to P. pestis are shown in Tables 1 and 2. Table 1 shows that cell yield and growth temperature share an inverse relationship. A more direct relationship is indicated between growth temperature and virulence, with the higher temperatures favoring virulence in the organism. Growth on MGOX agar is comparable at all three temperatures, as is the percentage pigmented on hemin agar.

Table 2 shows dose and MTD for the graded response test. The results are presented graphically in Figure 1, which shows that virulence of P. pestis can be reliably determined by the graded response in 10 to 40 hours, far less time than the 10 days ordinarily required for the quantal response. As with the quantal response test, determinations by the graded response show the greatest virulence and the least variability in response in cultures grown at higher temperatures. Variability of response of cultures grown at 26 C is reflected not only in wider confidence limits in the quantal test but also in the degree of scatter of the points on the slope for the graded test. This variability is of some importance since, according to the literature, most cultures are grown at 26 to 28 C to obtain greater cell yield and in the belief that cultures are more stable at these lower temperatures.

TABLE 1. VIABLE CELL COUNT, PER CENT DIFFERENTIAL RESPONSE
ON INDICATING MEDIA, AND LD₅₀ OF *P. PESTIS* GROWN
AT DIFFERENT TEMPERATURES

Growth Temperature, C	Total Viable Cell Count, 10 ⁸ /ml	Count on MGOX Agar, % of total	Pigmented on Hemin Agar, % of total	LD ₅₀ cells
26	346	1.21	74.6	6.7 (3.1-14.6) ^{a/}
31	202	3.96	75.0	5.5 (3.0-9.9)
37	45.6	2.85	73.3	0.6 (0.3-1.3)

a. 95% confidence limits.

TABLE 2. DOSE AND MEDIAN TIME TO DEATH OF MICE CHALLENGED
WITH *P. PESTIS* GROWN AT DIFFERENT TEMPERATURES

Growth Temperature					
26 C		31 C		37 C	
Log Dose	MTD, hr	Log Dose	MTD, hr	Log Dose	MTD, hr
9.238	<6.8	9.004	<6		
8.238	12.4	8.004	11.6	8.358	8.0
7.238	30.7	7.004	20.6	7.358	10.9
6.238	43.8	6.004	26.8	6.358	19.5
5.238	39.8	5.004	30.1	5.358	27.2
4.238	41.9	4.004	39.3	4.358	31.3
3.238	67.8	3.004	45.0	3.358	39.7
2.238	58.3	2.004	56.6	2.358	45.6
1.238	^{a/}	1.004	91.1	1.358	56.0
0.238	^{a/}	0.004	^{a/}	0.358	113.0

a. Partial response - not calculable.

After partial slope has been established by the graded test for a particular set of experimental conditions, virulence level may be determined with a minimum of five animals. This reduction in number of animals required for the graded test, as well as the shorter holding time makes this test considerably more economical than the quantal

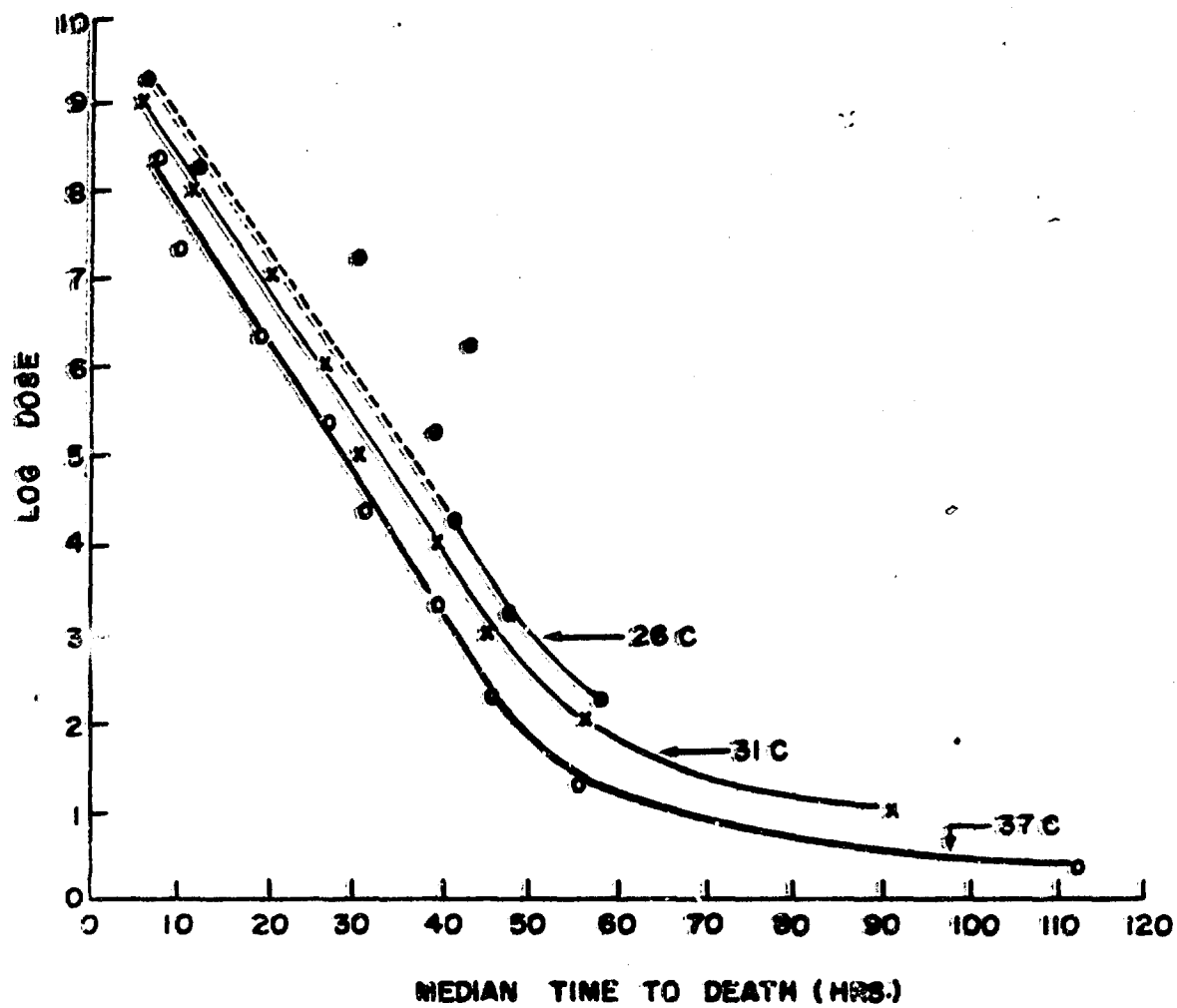


Figure 1. Dose and Graded Response of Mice to *P. pestis* Grown at Different Temperatures.

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