

Survivor Curves for *Leptospira autumnalis* Akiyami A Based on Most-Probable-Number Values

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The validity of survivor curves for *Leptospira autumnalis* Akiyami A based on most-probable-number values is supported by the following observations: (i) linear regression lines fell within most of the 95% confidence intervals; (ii) linear correlation coefficients (r) were consistently high (i.e., near -1); and (iii) statistical tests for goodness of fit usually accepted the linear model. These tests are consistent with an exponential death rate for the test organism in defined solutions. The influence of temperature and pH on survival was demonstrated by showing a statistically significant difference in survivor curve slopes.

It was previously demonstrated that a single cell of *Leptospira autumnalis* Akiyami A could be recovered with a supplemented Fletchers medium (Difco) and that the density of washed-cell suspensions could be estimated by the most-probable-number (MPN) procedure (4). This report concerns an investigation of the applicability of the MPN procedure for generation of survivor curves.

MATERIALS AND METHODS

Test organism. The test strain of *L. autumnalis* Akiyami A was obtained from A. D. Alexander, Water Reed Army Medical Center. The organism was maintained in Fletchers medium (Difco) containing 10% rabbit serum (Pel-Freez) with transfer every 5 weeks. Antigenic stability was verified periodically with antiserum provided by the Center for Disease Control.

Preparation of cells. A 1-ml amount of stock culture was used to inoculate 10 ml of a medium containing (per liter): Na_2HPO_4 , 1.0 g; KH_2PO_4 , 0.3 g; NaCl, 1.0 g; NH_4Cl , 0.25 g; thiamine, 0.005 g; and rabbit serum, 100 ml. After incubation at 30 C for 86 hr, the cells were removed by centrifugation at 3,000 rev/min for 30 min. The cells were washed twice with 5 ml of buffer (5.33 mM phosphate, pH 7.6) and resuspended in 10 ml of buffer. Cell concentration was standardized at 10^8 per ml by direct count with a Petroff-Hausser chamber and dark-field microscopy. A 1-ml amount of the standardized suspension was added to 100 ml of test solution to give an initial cell concentration of 990 per ml.

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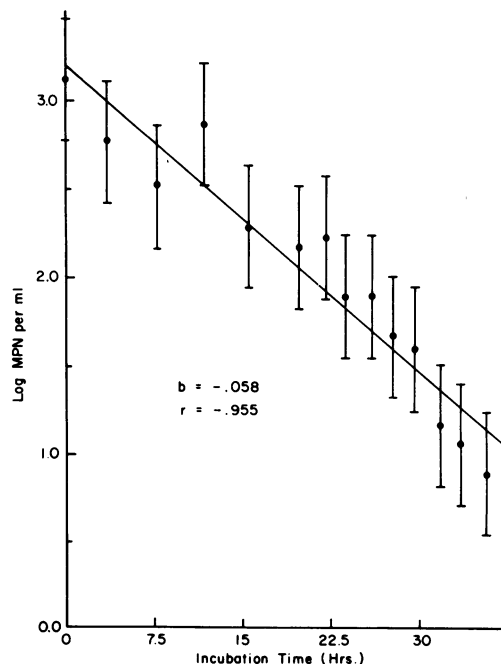


FIG. 1. Survival of *Leptospira autumnalis* in Ringer's solution at pH 7.10 and 20 C. MPN values (10 tubes per dilution) shown with their 95% confidence intervals; b = slope; r = linear correlation coefficient.

Test solutions. The composition of test solutions was based on previous efforts to find a simple, defined system which would support survival for a

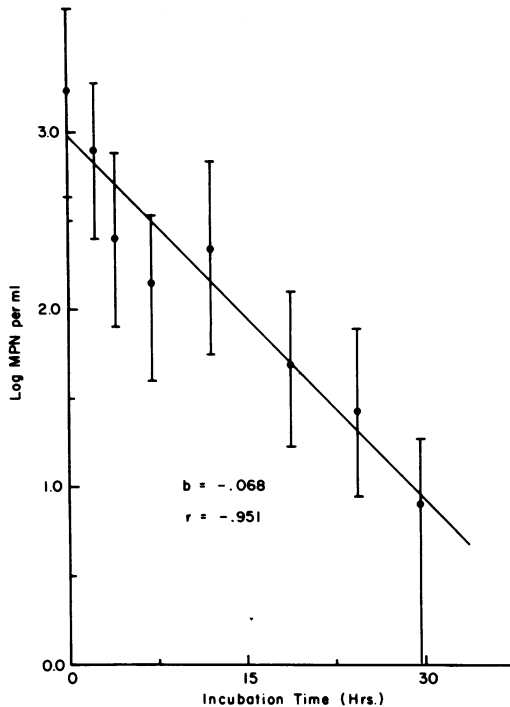


FIG. 2. Survival of *Leptospira autumnalis* in Ringer's solution at pH 7.15 and 20 C. MPN values (five tubes per dilution) shown with their 95% confidence intervals; b = slope; r = linear correlation coefficient.

time long enough to permit study of the influence of other variables. The survivor curves presented here were obtained with two test solutions, Ringer's solution and sodium thiosulfate (4.95 mM), each buffered at a final phosphate concentration of 5.33 mM. Ringer's solution contained (per liter): NaCl, 537.5 mg (9.19 mM); KCl, 18.75 mg (0.25 mM); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 39.7 mg (0.273 mM); and $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 125 mg (0.504 mM).

To study the influence of pH in solutions of sodium thiosulfate (4.95 mM), the concentration of buffer was increased to 10 mM total phosphate.

Test solutions were prepared with water passed through an ion-exchange column and glass-distilled. Thiosulfate concentrations were determined by titration with standard iodine solution. Test solutions were sterilized by filtration through a 0.22- μm membrane filter (Millipore Corp.), and 100 ml of solution was transferred to a sterile cotton-stoppered 250-ml Erlenmeyer flask. After inoculation, the flasks were held static in the dark at 20 C unless temperature was a variable.

MPN procedure. Dilutions for MPN determinations were made in 9.0-ml blanks of *Leptospira* medium EMJH (Difco) containing 1% rabbit serum. Amounts of 0.1 ml of three decimal dilutions were transferred to three series of 5 or 10 tubes each (plastic, 12 by 75 mm, Falcon Plastics) containing 4.0 ml of Fletchers medium (Difco) supplemented with

(per liter): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg; asparagine, 150 mg; and rabbit serum (Pel-Freez), 100 ml. Filter-sterilized asparagine and rabbit serum were added aseptically to autoclaved medium before dispensing.

Incubation was at 30 C for up to 17 days. Positive tubes were those showing visible evidence of growth, usually obvious by the characteristic Dinger's ring of *Leptospira*. MPN values were obtained from available tables.

Statistical tests. The goodness-of-fit test for a linear model was completed by calculating the chi-square test statistic given by:

$$X^2 = \frac{s^2 (\text{d.f.})}{\sigma^2}$$

where s^2 = residual mean square; d.f. = degrees of freedom = $m - 2$; m = number of time points; $\sigma^2 = (0.5487/\sqrt{n})^2$; and n = number of tubes per dilution. The variance (σ^2) is based on the accepted value for the standard deviation of log MPN (6, 7) and is the expected value of the residual mean square (s^2).

$$\sigma_{\log} = \frac{0.5487}{\sqrt{n}}$$

If the calculated value of X^2 was greater than that from the chi-square distribution for $m - 2$ degrees of

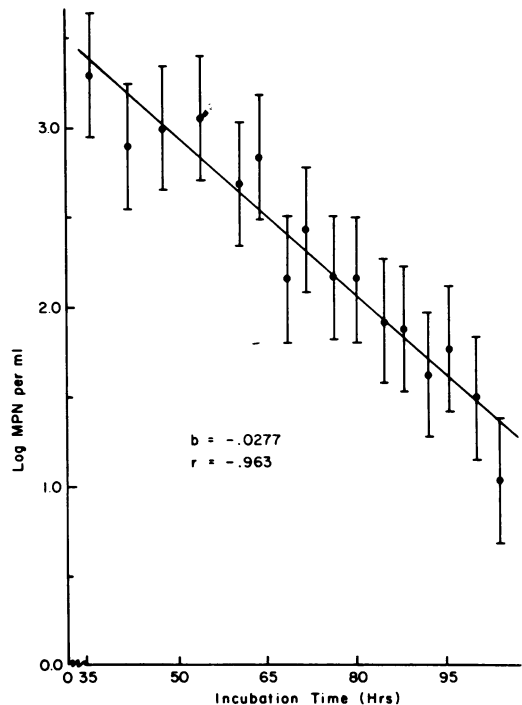


FIG. 3. Survival of *Leptospira autumnalis* in a buffered (5.33 mM phosphate) thiosulfate (4.95 mM) solution at pH 7.39 and 20 C. MPN values (10 tubes per dilution) shown with their 95% confidence intervals; b = slope; r = linear correlation coefficient.

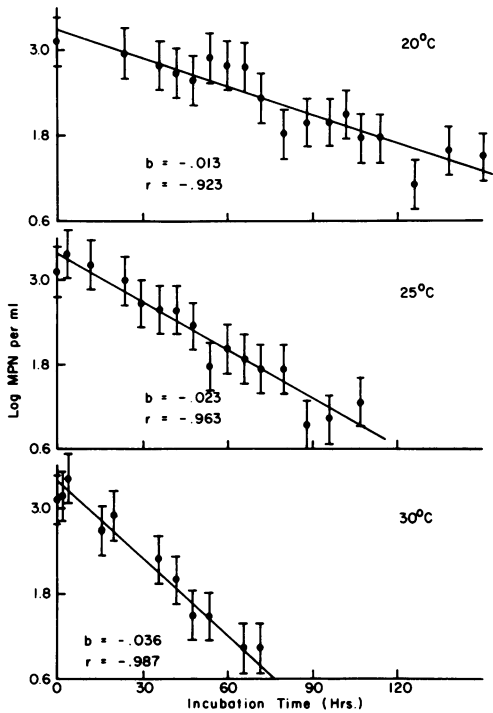


FIG. 4. Effect of temperature on survival of *Leptospira autumnalis* in buffered (5.33 mM phosphate) thiosulfate (4.95 mM) solutions at pH 7.40. MPN values (10 tubes per dilution) shown with their 95% confidence intervals; b = slope; r = linear correlation coefficient.

freedom and $\alpha = 0.05$, then the linear model was rejected.

Slopes were tested for significant difference by calculating the chi-square test statistic given by:

$$X^2 = \frac{(b_1 - b_2)^2}{s_1^2 + s_2^2}$$

where b_1 = slope of curve 1; b_2 = slope of curve 2; s_1^2 = variance of curve 1; and s_2^2 variance of curve 2. If the calculated value of X^2 was greater than that from the chi-square distribution for one degree of freedom and $\alpha = 0.05$, then the hypothesis of equal slopes was rejected.

RESULTS

Figure 1 presents a survivor curve for *L. autumnalis* in Ringer's solution at pH 7.10 and 20 C. MPN values are based on 10 tubes per dilution and are plotted with their 95% confidence intervals. The linear regression line is presented as the best straight-line fit of the data. Figure 2 shows a similar survivor curve based on fewer MPN values with five tubes per dilution. The range of the 95% confidence interval increases as the number of tubes per

dilution decreases. Figure 3 shows a survivor curve for *L. autumnalis* in a buffered thiosulfate solution. The concentration of sodium thiosulfate (4.95 mM) is about 10 times greater than in Ringer's solution (0.504 mM), producing

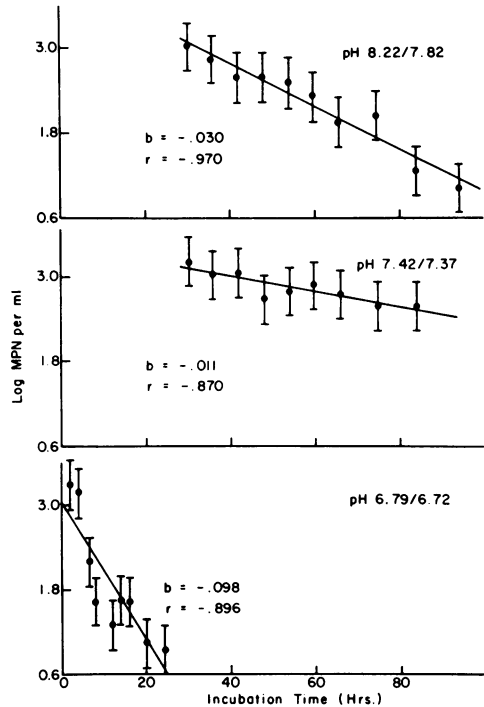


FIG. 5. Effect of pH on survival of *Leptospira autumnalis* in buffered (10 mM phosphate) thiosulfate (4.95 mM) solutions at 20 C. The pH is given as initial/final. MPN values (10 tubes per dilution) shown with their 95% confidence intervals; b = slope; r = linear correlation coefficient.

TABLE 1. Goodness of fit of linear model for *Leptospira autumnalis* survivor curves

Determination	Test conditions ^a		
	Ringer's solution		Na ₂ S ₂ O ₃ , 4.95 mM, pH 7.39
	pH 7.10	pH 7.15	
No. of tubes per dilution for MPN, n . . .	10	5	10
No. of time points, m . . .	14	8	16
Degrees of freedom (d.f.), $m - 2$	12	6	14
Residual mean square, s^2	0.044700	0.056604	0.033218
χ^2 ($m - 2$ d.f., $\alpha = 0.05$)	21.03	12.59	23.68
χ^2 , calculated	17.82	5.64	15.45
Goodness of fit	Accept	Accept	Accept

^a All tests were at 20 C.

TABLE 2. Goodness of fit of linear model for *Leptospira autumnalis* survivor curves at different pH

Determination	pH (initial/final)		
	6.79/6.72	7.42/7.37	8.22/7.82
No. of time points, m	10	9	10
Degrees of freedom (d.f.), $m - 2$	8	7	8
Residual mean square, s^2	0.16842	0.013430	0.027967
χ^2 ($m - 2$ d.f., $\alpha = 0.05$)	15.51	14.07	15.51
χ^2 , calculated	44.76	3.13	7.43
Goodness of fit	Reject	Accept	Accept

TABLE 3. Goodness of fit of linear model for *Leptospira autumnalis* survivor curves at different temperature

Determination	Temp (C)		
	20	25	30
No. of time points, m	18	16	13
Degrees of freedom (d.f.), $m - 2$	16	14	11
Residual mean square, s^2	0.054615	0.046420	0.036125
χ^2 ($m - 2$ d.f., $\alpha = 0.05$)	26.30	23.68	19.68
χ^2 , calculated	29.03	21.59	13.20
Goodness of fit	Reject ^a	Accept	Accept

^a Would accept at $\alpha = 0.01$.

a lower death rate (b).

Figure 4 shows the influence of temperature on survival of *L. autumnalis* through differences in the slopes of survivor curves. Temperatures above 20 C increased the death rate. The effect of pH shown in Fig. 5 indicates that pH values below and above 7.4 increased the death rate, with a greater sensitivity to acid pH values.

A statistical test for goodness of fit on the survivor curves presented in Fig. 1, 2, and 3 accepts a linear model at $\alpha = 0.05$ (Table 1). The same test rejects a linear model for one of the pH survivor curves (Table 2) and one of the temperature survivor curves (Table 3). The temperature curve would be acceptable at $\alpha = 0.01$.

Statistical analyses for significant difference in slopes between each pair of pH survivor curves and each pair of temperature survivor curves rejects a hypothesis of equal slopes at $\alpha = 0.05$. In other words, each of the pH curves has a slope significantly different from the other two curves, and the same is true for the

temperature curves.

DISCUSSION

The validity of the survivor curves for *L. autumnalis* based on MPN values is supported by the following observations. (i) The linear regression line for log MPN versus time fell within most of the 95% confidence intervals; the nine linear regression lines presented missed 9 of 114 (7.9%) confidence intervals. (ii) The linear correlation coefficient was consistently high, i.e., near -1 (range: -0.870 to -0.987); this coefficient is a measure of the strength of the linear relationship between the variables. (iii) A statistical test for goodness of fit usually accepted a linear model for log MPN versus time.

These three tests are consistent with an exponential death rate in the test solutions and support the reliability of the MPN procedure for estimating the number of survivors.

The influence of temperature and pH on survival was demonstrated through statistical analyses for difference in survivor curve slopes. The results generally agree with other reports on the effect of temperature (2, 3) and pH (1, 5) on survival of *Leptospira*. The results substantiate the sensitivity of this method for studying the influence of environmental factors on survival. The entire procedure is offered as a more quantitative model for survival studies with *Leptospira*.

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