

Statistical Characterization of Negative Control Data in the Ames Salmonella/Microsome Test

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A statistical characterization of negative control data in the Ames Salmonella/microsome reverse mutation test was performed using data obtained at Takeda Analytical Research Laboratories during January 1989 to April 1990. The lot-to-lot variability of bacterial stock cultures and day-to-day variability of experiments were small for *Salmonella typhimurium* strains TA1535 and TA1537 and *Escherichia coli* WP2uvrA, but they were larger for *S. typhimurium* TA100. The number of revertant colonies for all test strains studied here followed Poisson distributions within the same day. The two-fold rule that is an empirical method to evaluate the Ames Salmonella/microsome test results has been widely used in Japan. This two-fold rule was evaluated statistically. The comparison-wise type I error rate was less than 0.05 for TA98, TA100, TA1535, TA1537, and WP2uvrA. Moreover, this rule is particularly conservative for TA100, for which the type I error rate was nearly 0.

Introduction

The Ames Salmonella/microsome test (*Escherichia coli* WP2uvrA is also included in the present study) is commonly used to evaluate the mutagenicity of chemicals and potential drugs, and many statistical procedures for analyzing the test results have been proposed. These procedures were reviewed, and a recommended method was selected by the United Kingdom Environmental Mutagen Society (1). In Japan, however, the empirical two-fold rule is widely used. In the Guidelines for Toxicity Studies of Drugs (2), the evaluation of test results is described as follows: "The test substance is considered to be positive for mutagenic activity when the number of revertant colonies per plate with the test substance is more than twice that per negative control plate and, in addition, when a dose-related increase in mutation count is observed."

The aims of the present study were to a) evaluate the lot-to-lot variability of bacterial stock cultures, day-to-day variability of experiments, and also plate-to-plate variability in the Ames Salmonella/microsome test; b) determine whether the distribution of frequencies of revertant colonies follows Poisson distributions; and c) evaluate the two-fold rule statistically.

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Data Used for Analysis

The test strains considered in the present study were *Salmonella typhimurium* TA1535 and TA100 and *Escherichia coli* WP2uvrA for base substitution and *S. typhimurium* TA1537 and TA98 for frameshift mutation. *Escherichia coli* WP2uvrA was obtained from T. Matsushima of the University of Tokyo, and all other strains were obtained from B. N. Ames of the University of California, Berkeley. Small portions of stock cultures containing 8% dimethyl sulfoxide (DMSO) were stored in polypropylene tubes at less than -80°C . These were used as the seed for culture of the test strain at an interval of about 6 months. For the exogenous metabolic activation system (S9 mix), the post-mitochondrial fraction of sodium phenobarbital and β -naphthoflavone pretreated rat liver homogenate supplemented with cofactors was used. Bacteria, solvent (distilled water or DMSO) and S9 mix (or phosphate buffer for nonmetabolic activation) were mixed and preincubated at 37°C for 20 min. Revertant colonies on agar plates were counted using an electronic colony counter after 48 hr of incubation at 37°C .

Two different kinds of negative control data were used for analysis. One was the historical control data obtained from the duplicate, negative control plates in the course of routine work during January 1989 to April 1990 at Takeda Analytical Research Laboratories. The other was data obtained from 50 replicate solvent negative control plates within the same day.

Methods for Analysis

To examine sources of variability in the negative control data, differences among the lots of bacterial stock cultures, among the days when experiments were performed, and also among the

plates within the same day were studied using the SAS System (3,4). The UNIVARIATE, GLM, NESTED, and VARCOMP procedures were used. Analysis of variance (ANOVA) for the historical control data was performed based on the following nested model (Eq. 1).

$$y_{ijk} = \mu + L_i + D(L)_{ij} + R(DL)_{ijk} \quad (1)$$

with

$$\begin{aligned} y_{ijk} &= \text{colony count} \\ i &= \text{lot of bacterial seed} = 1,2,3 \\ j &= \text{day} = 70, \dots, 84 \\ k &= \text{plate} = 1,2(4) \end{aligned}$$

L indicates lot factor with variance component σ_L^2

D indicates (inter-)day factor with variance component σ_D^2

R indicates plate factor with (intra-day)

variance component σ_R^2

where the variable y_{ijk} is the colony count of lot i , day j , and the k th plate, and the parameters σ_L^2 , σ_D^2 , and σ_R^2 denote the variance component among lots and the inter- and intra-day difference, respectively.

The Poisson assumption of the distribution of colony count in the Ames Salmonella/microsome test was tested by means of the goodness of the fit of four Poisson models (a, b, c, and d) to the data (5).

Model a (common parameter):

$$E[y_{ijk}] = \mu \quad \text{for all } i,j,k \quad (2)$$

with

$$\chi^2 = \sum_i \sum_j \sum_k \frac{(y_{ijk} - \bar{y}_{\dots})^2}{\bar{y}_{\dots}}$$

$$\phi = \left(\sum_i \sum_j n_{ij} \right) - 1$$

Model b (lot-to-lot differential parameter):

$$E[y_{ijk}] = \mu_i \quad \text{for all } i,j,k \quad (3)$$

with

$$\chi^2 = \sum_i \sum_j \sum_k \frac{(y_{ijk} - \bar{y}_{i..})^2}{\bar{y}_{i..}}$$

$$\phi = \sum_i \left\{ \left(\sum_j n_{ij} \right) - 1 \right\}$$

Model c (day-to-day differential parameter):

$$E[y_{ijk}] = \mu_{ij} \quad \text{for all } i,j,k \quad (4)$$

with

$$\chi^2 = \sum_i \sum_j \sum_k \frac{(y_{ijk} - \bar{y}_{ij.})^2}{\bar{y}_{ij.}}$$

$$\phi = \sum_i \left\{ \sum_j (n_{ij} - 1) \right\}$$

Model d (one parameter):

$$E[y_k] = \mu \quad \text{for all } k \quad (5)$$

with

$$\chi^2 = \sum_k \frac{(y_k - \bar{y})^2}{\bar{y}}$$

$$\phi = n - 1$$

and

$$y = \text{colony count}$$

$$n = \text{number of replicate plates}$$

$$\phi = \text{degrees of freedom}$$

Models a, b, and c were applied to the historical control data including three lots of each test strain obtained during a period of 70–84 days. Model d was applied to 50 replicate plates to assess the intra-day distribution.

The type I error of the empirical two-fold rule was evaluated by the following procedures. Equation 6 is the experimentwise error rate under the complete null hypothesis, which is the probability that at least one mean colony count of duplicate plates among $k-1$ treated groups is equal to or greater than twice that in the concurrent negative control.

$$\alpha \int_0^{\infty} f(x) \{1 - F(2x)\}^{k-1} dx \quad (6)$$

where

k = number of groups, including negative control

x = mean colony counts of duplicate plates per group

$f(x)$ = probability density function of x

$F(x)$ = distribution function of x

The distribution of x (mean colony counts of duplicate plates) is evaluated based on the distribution of y (individual colony counts), assuming both theoretical (Poisson and negative binomial distributions) and empirical (the data obtained from the experiments using 50 replicate plates) distributions.

Results

The number of plates, the mean of revertant colonies per plate, and the variance for each test strain for historical data (inter-day) and 50 replicate plate data (intra-day) are summarized in Table 1. The mean colony counts were liable to large variation among strains; for example, TA1535 showed less than 10 colonies per plate on the average, but TA100 showed more than 100. The intra- and inter-day variances for all strains were more or less comparable to their mean, except for the inter-day variances of TA100, which showed overdispersion, i.e., the variances were about five times greater than the mean.

To examine the contribution of each source of variability, lot, day, and plate, ANOVA was performed for the historical control data (Table 2). Table 2 shows the relative percentages of the variance components for lot, day, and plate. The patterns of contribution of these sources of variability to the variance were

Table 1. Summary statistics of the two types of negative control data.

Species	Experimental conditions			Historical data			50 Replicate plates		
	Strain	S9 mix	Solvent	n	Mean	Variance	n	Mean	Variance
<i>E. coli</i>	WP2uvrA	+	DW	162	15.2	18.1	50	17.0	12.6
		+	DMSO	190	13.9	15.8	50	14.3	10.4
		-	DW	142	12.3	14.3	50	12.6	9.9
		-	DMSO	146	10.4	9.6	50	11.5	13.0
<i>S. typhimurium</i>	TA100	+	DW	162	128.4	850.4	50	149.0	210.2
		+	DMSO	190	105.9	643.0	50	111.6	109.9
		-	DW	142	143.9	599.0	50	154.9	143.5
		-	DMSO	146	132.5	757.8	50	140.5	128.4
	TA1535	+	DW	162	7.7	9.5	50	7.2	5.7
		+	DMSO	190	6.6	8.5	50	6.8	6.0
		-	DW	142	7.2	7.4	50	7.1	6.1
		-	DMSO	146	7.0	6.2	50	7.1	9.0
	TA98	+	DW	162	26.7	26.2	50	30.8	39.7
		+	DMSO	190	27.2	34.3	50	31.7	38.0
		-	DW	142	37.2	128.1	50	41.6	64.7
		-	DMSO	146	29.3	53.5	50	32.6	27.9
	TA1537	+	DW	162	8.7	12.7	50	7.5	6.3
		+	DMSO	190	8.8	10.2	50	8.3	6.1
		-	DW	142	9.8	15.3	50	14.5	17.2
		-	DMSO	146	9.0	15.5	50	12.5	11.6

Abbreviations: DW, distilled water; DMSO, dimethyl sulfoxide

different among strains and also depended on the presence or absence of S9 mix. It is notable that the lot-to-lot and day-to-day variations of TA100 are larger than those of other strains. In contrast, the lot-to-lot and day-to-day variations of WP2uvrA were found to be much less than the plate-to-plate variation.

The Poisson assumption was evaluated for each test strain using models a, b, and c for historical data and using model d for 50 replicate plates. Results are summarized in Table 3. Table 3 shows that models a and b do not fit the data except for that of strain WP2uvrA. Although model c was rejected twice, it was considered to be valid because independent statistical tests were applied 20 times for each model. This suggests that the assumption of Poisson distribution is reasonable for data obtained within the same day.

The values of theoretical type I error of the two-fold rule are summarized in Table 4 assuming Poisson and negative binomial distributions. Table 4 shows that when expected colony counts increase, the type I error rate decreases. Under the Poisson assumption, when the expected values of revertant colonies per plate are more than 10, the type I error rate decreases to less than 5% in the two-group experiment. Therefore, when the number of the revertant colonies per plate is more than 50 in the case of TA100, the type I error rate is substantially zero. Under the assumption of negative binomial distribution, which has larger variance than Poisson distribution, type I error rates are greater than with Poisson distribution. Generally, the six-group experiment data showed a larger type I error rate than the two-group experiment data.

Table 5 shows values of type I error based on empirical distribution of the 50 replicate plates. The values of type I error for strain TA1535 were the highest among the five strains studied but were still less than or nearly equal to 5%. The values of type I error for other strains, especially TA100 and TA98,

Table 2. The variance components in the historical control data.

Species	Experimental conditions			Relative percentage*		
	Strain	S9 mix	Solvent	Lot	Day	Plate
<i>E. coli</i>	WP2uvrA	+	DW	12.6	11.4	76.0
		+	DMSO	8.1	11.6	80.3
		-	DW	0.0	19.1	80.9
		-	DMSO	1.0	1.6	97.4
<i>S. typhimurium</i>	TA100	+	DW	37.8	45.0	17.2
		+	DMSO	39.8	43.5	16.6
		-	DW	61.1	15.7	23.2
		-	DMSO	47.7	28.8	23.4
	TA1535	+	DW	2.5	23.1	74.5
		+	DMSO	3.1	35.9	61.0
		-	DW	0.0	5.0	95.0
		-	DMSO	0.6	2.2	97.2
	TA98	+	DW	19.6	24.1	56.3
		+	DMSO	16.7	6.6	76.7
		-	DW	45.1	29.7	25.2
		-	DMSO	28.7	31.9	39.3
TA1537	+	DW	2.1	30.3	67.6	
	+	DMSO	5.2	18.8	76.0	
	-	DW	12.9	9.5	77.6	
	-	DMSO	8.7	25.5	65.8	

Abbreviations: DW, distilled water; DMSO, dimethyl sulfoxide.

*Relative percentage = [(each estimate of variance component)/($\hat{\sigma}_L^2 + \hat{\sigma}_D^2 + \hat{\sigma}_R^2$) X 100]

TA100 and TA98, were much less than 5%. These results indicate that although the two-fold rule has no theoretical basis, the values of type I error are smaller than 5%, the commonly used statistical significance level.

Table 3. The validity of the Poisson assumption

Experimental conditions				Evaluation of Poisson assumption (χ^2/ϕ) ^a			
Species	Strain	S9 mix	Solvent	Historical data ^b			50 replicate plates ^c
				Model a	Model b	Model c	Model d
<i>E. coli</i>	WP2uvrA	+	DW	1.19*	1.08	0.92	0.74
		+	DMSO	1.14	1.09	0.97	0.73
		-	DW	1.16	1.17	0.92	0.78
		-	DMSO	0.92	0.92	0.84	1.14
<i>S. typhimurium</i>	TA100	+	DW	6.62 ⁺	4.72 ⁺	1.14	1.41*
		+	DMSO	6.07 ⁺	4.11 ⁺	1.22	0.99
		-	DW	4.16 ⁺	2.12 ⁺	1.19	0.93
		-	DMSO	5.72 ⁺	3.57 ⁺	1.57 ⁺	0.91
	TA1535	+	DW	1.22*	1.21*	0.93	0.79
		+	DMSO	1.29 ⁺	1.25*	0.83	0.89
		-	DW	1.01	1.02	0.98	0.86
		-	DMSO	0.89	0.88	0.87	1.27
	TA98	+	DW	0.98	0.86	0.63	1.29
		+	DMSO	1.26 ⁺	1.12	0.99	1.20
		-	DW	3.44 ⁺	2.42 ⁺	1.14	1.56 ⁺
		-	DMSO	1.83 ⁺	1.54 ⁺	0.88	0.86
TA1537	+	DW	1.46 ⁺	1.45 ⁺	1.08	0.84	
	+	DMSO	1.17	1.11	0.91	0.73	
	-	DW	1.57 ⁺	1.44 ⁺	1.31*	1.19	
	-	DMSO	1.72 ⁺	1.62 ⁺	1.16	0.93	

Abbreviations: DW, distilled water; DMSO, dimethyl sulfoxide.

^a χ^2 , chi-square statistics for the test of Poisson distribution; ϕ , degree of freedom.

^bModel a, common parameter model; model b, lot-to-lot differences parameter model; model c, day-to-day differences parameter model.

^cModel d, one-parameter model.

* $p < 0.05$.

⁺ $p < 0.01$.

Table 4. Type I error (α) of the two-fold rule: theoretical distribution (%).

μ [E (Y)]	Poisson		Negative binomial ^a		
	2 Groups	6 Groups	σ^2	2 Groups	6 Groups
5	8.42	20.18	6.25	10.85	25.34
10	2.15	6.31	15.00	4.81	13.21
15	0.61	2.02	26.25	2.76	8.31
20	0.18	0.65	40.00	1.84	5.86
25	0.05	0.21	56.25	1.34	4.46
50	0.00	0.00	175.00	0.55	2.02
100	0.00	0.00	600.00	0.28	1.10

^a $\sigma^2 = \mu (1 + 0.05\mu)$.

Discussion

The present study showed that the application of the Poisson assumption to the Ames Salmonella/microsome test data obtained within the same day was generally acceptable. Therefore, statistical procedures based on Poisson distribution (*I*), such as a likelihood ratio test, can be applied to the evaluation of the test results. This means that statistical procedures that presume overdispersion are not necessary, although many sophisticated statistical models that take overdispersion into consideration (6-9) have been proposed in the field of mutagenicity testing.

It is notable that the values of type I error of the two-fold rule are almost always below 5%, and using duplicate plates is sufficient to reduce the type I error rate. The application of the two-fold rule to the data obtained with strain TA100 might be too conservative.

In conclusion, we believe that if an experiment is carried out

Table 5. Type I error (α) of the two-fold rule: empirical distribution based on 50 replicate plates.

Experimental conditions				Type I error (%)	
Species	Strain	S9 mix	Solvent	2 Groups	6 Groups
<i>E. coli</i>	WP2uvrA	+	DW	0.05	0.22
		+	DMSO	0.09	0.42
		-	DW	0.55	1.70
		-	DMSO	2.44	6.20
<i>S. typhimurium</i>	TA100	+	DW	0.00	0.00
		+	DMSO	0.00	0.00
		-	DW	0.00	0.00
		-	DMSO	0.00	0.00
	TA1535	+	DW	2.33	7.16
		+	DMSO	4.86	10.87
		-	DW	3.21	9.40
		-	DMSO	5.49	18.17
	TA98	+	DW	0.01	0.03
		+	DMSO	0.01	0.07
		-	DW	0.02	0.08
		-	DMSO	0.00	0.00
TA1537	+	DW	2.46	7.82	
	+	DMSO	1.11	4.09	
	-	DW	1.67	4.03	
	-	DMSO	0.97	2.91	

Abbreviations: DW, distilled water; DMSO, dimethyl sulfoxide.

carefully to eliminate sources of variability as completely as possible, the data from the Ames Salmonella/microsome test follow Poisson distributions. A sophisticated and complicated

statistical model is therefore not necessarily required to evaluate the test results. The two-fold rule is acceptable from the viewpoint of type I error rate, but could be too conservative; this rule might be improved by incorporating a method for evaluating the dose-response relationship.

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