

*Lamivudine-resistant HIV Type 1 Clinical Isolates  
Under Lamivudine Pressure  
Show Impaired Selection of Resistance  
to Nucleoside Reverse Transcriptase Inhibitors*

*by*

*Barbara A Rath, Richard A. Olshen, Jerry Halpern,  
Christian R. Frey, Suzan L. Buge, and Thomas C. Merigan*

## APPENDICES

**A. Viral Particle Model.** One approach to testing differences at two fixed passages between two isolates, neither descended from the other, was by what we have termed the *viral particle model*. Background is given in *Statistical Methods* and applications in **RESULTS**. The basis for comparison at a fixed codon is a two-sample t-like statistic that is the difference of two fractions divided by an estimate of the standard deviation of that difference. We denote the two isolates by  $a$  and  $b$ . For  $a$ , say, the indicator of particle  $i$  having its baseline value at codon  $c$  is  $I_i(c)$ , which has value 1 if  $c$  is wild type, and otherwise is 0. There is an analogous indicator for  $b$ . As in the body of the paper we take the numbers of viral particles to be 30,000. We speak of the correlation  $\rho$  between two indicators. For two codons  $i$  and  $i'$  we write this correlation  $\rho(i, i')$ . What matters is actually the value of  $\rho(i, i')$  averaged over pairs  $(i, i')$  of codons. From the constraint that the variance of a sum cannot be negative, it follows easily that the average  $\rho(i, i')$  cannot be less than  $-3.33 \times 10^{-5}$ . In fact we expect that  $\rho$ , which we cannot know exactly, is positive and small. Because two particles within the same isolate and passage may have replicated inside the same cell, and also because of the physical proximity of any two particles within a flask can vary, we do not assume *a priori* that  $\rho = 0$ .

For a comparison of differences, the numerator of the t-like statistic is

$$\hat{p}_a - \hat{p}_b, \quad \text{where } \hat{p}_a = (1/30,000) \sum_{i=1}^{30,000} I_i(c),$$

and  $\hat{p}_b$  is defined by analogy. From a well-known computation with sums of random variables that assume only values 0 and 1 it follows that the variance of  $\hat{p}_a$ ,

$$V(\hat{p}_a) = (p_a(1 - p_a)/30,000) + ((29,999/30,000)\rho p_a(1 - p_a)), \quad (A.1)$$

where,

$$p_a = E(I_i(c)) = Prob(I_i(c) = 1).$$

Note that this probability is assumed here not to depend on  $i$ . (Of course, computations that follow in this appendix, and that are required elsewhere in the paper, show this assumption to be false, decisively. However, the net effect of our assumptions is to make the p-values of our test extremely conservative.) We estimate  $V(\hat{p}_a)$  by replacing  $p_a$  on the right hand side of (A.1) by  $\hat{p}_a$ .  $V(\hat{p}_b)$  is estimated analogously. Because  $\hat{p}_a$  and  $\hat{p}_b$  are clearly independent, our t-like statistic is now seen to be

$$t_c = \frac{\hat{p}_a - \hat{p}_b}{\sqrt{\hat{V}(\hat{p}_a) + \hat{V}(\hat{p}_b)}}$$

The missing ingredient in  $t_c$  is  $\rho$ , which we admittedly have no way of knowing exactly. But for that, we could approximate p-value for testing the null hypothesis “no difference between given isolates and passages at codon  $c$ ” by  $Prob(|Z| > t)$ , where  $Z$  is a standard Gaussian random variable; and  $t$  the observed value of  $t_c$ . We could then test the null hypothesis “no difference at any codon” by  $300Prob(|Z| > t)$ . The latter computation uses the simple Bonferroni bound. In fact what we wish to do with  $t_c$  is to find and use the largest value of  $\rho$  for which the cited 80% - 20% difference at some codon for fixed isolates and passages is significant at the 5% level for the null hypothesis as given. First, we solve  $Prob(|Z| > t) = .05/300$  for  $t$ , arriving at  $t = 3.7482$ . Then, set  $\hat{p}_a = .8$ ,  $\hat{p}_b = .2$ , and  $t_c = 3.7482$ ; and solve for  $\rho$ . The resulting  $\rho$  is .08.

**B. Significance of NNRTI Binding Pocket Mutations.** It was noted in **DISCUSSION** that for the 19 isolates for which treatment included escalating doses of *NVP*, there were 38 NNRTI binding pocket mutations. Of these, 13 were at codon 106, seven at 181, and five at 108. The p-values for the findings that the “most popular” codon of 11 had at least 13 mutations, alternatively that the “second most popular” had at least 11, under the

common null hypothesis that codons are “equidistributed” (exchangeable) were computed thus.

Isolates are taken to be independent, codons within isolates chosen at random without replacement from among the 11. Reading from isolate #1 through #5, successively from *NVP* only through *NVP+3TC+ADV*, the respective numbers of NNRTI binding pocket mutations were seen to be 2,1,3,2,1,1,2,1,2,1,1,3,2,3,1,2,2,3,3. Therefore, the number of ways NNRTI binding pocket codons could be chosen is

$$\binom{11}{2} \binom{11}{1} \binom{11}{3} \binom{11}{2} \cdots \binom{11}{3} \binom{11}{3},$$

a product of 19 numbers. We made this choice at random 50,000 times, each time noting the codon chosen the largest, respectively next largest, number of times, thereby obtaining the joint sampling distribution of these two random quantities. Of the 50,000 trials, the “most popular” codon seen was seen only 12 times, and that occurred for only four trials. The “next most popular” was seen seven times (2,056 trials), eight times (129 trials), and nine times (seven trials). It follows that the respective estimated p-values are 1/50,001 (which is about 0.00002) and (2057+129+7)/50,001 (which is about 0.044) when the null hypotheses are as given. The first hypothesis seems untenable, and possibly not the second, either.

**C. Discussion of Two Further Models.** Numbers of mutations across isolates within a particular passage for a “counting process” such as ours might be taken to be what is conventional in such applications, a Poisson process. The Poisson model arises when there are many chances for “success” but few “successes,” and in addition trials are independent. These assumptions might apply when we take mutations themselves as sampling units. The presence of mutation or mutations within an isolate and drug combination can be assumed independent across passages; they are certainly independent across isolates. A sum of independent Poisson random variables has a Poisson distribution no matter the respective parameters of the summands. Conversely, if a sum of independent random variables has a Poisson distribution, then according to D. Raikov<sup>2</sup>, each summand has a

Poisson distribution. Therefore, we can test the null hypothesis that the Poisson model applies to numbers of mutations within a drug combination by looking at Passage 12 to see if the distribution of numbers of mutations across isolates is Poisson. We begin with the usual approach to assessing the Poisson model: via the “Poisson dispersion test”<sup>3</sup>. (In a Poisson model the mean and variance are equal as numbers.) The test statistic is proportional to the ratio of sample variance to sample mean. If the Poisson model for mutations were correct for a fixed treatment (or drug combination), but there was a change by isolate in the Poisson parameter with 184 reversal, then there would be evidence for over-dispersed data and thus evidence against a strict Poisson model. Other aspects of the experiment could lead to over-dispersed or under-dispersed numbers of mutations. (Over[under]-dispersion in a model means that the variance is greater [less] than what the model would constrain it to be.) There are some 0s in the sample variances when isolates are pooled within treatments. The ratio of sample variance to sample mean disregards information in the sample mean when the sample variance is 0. This observation and a Taylor series argument not given here led us to use as a test statistic the difference of sample mean and sample variance rather than the usual ratio. On the null hypothesis that the data are Poisson, the difference should be 0 to within noise. Because there are at most five isolates per drug combination, we could not rely on asymptotic distributions computed under the null hypothesis. Instead, we used the parametric bootstrap distribution<sup>4</sup> of the test statistic under the null hypothesis. This amounts to sampling independently from a Poisson distribution with parameter (mean and variance) the average number of mutations observed at the twelfth passage. The resulting distribution is the reference distribution for the cited difference when the null hypothesis is true. We took 1,000 bootstrap samples per drug combination. This approach enabled us to compute p-values for the null hypothesis separately for alternatives of over-dispersion and under-dispersion relative to the Poisson.

When the number of mutations across isolates within a particular passage is hypothesized to have a Poisson distribution and tested as specified, then the p-value for “over-dispersion” is never less than .85. However, for the model with “underdispersion,” the respective p-values are .137 for *NVP only*, .147 for *NVP+3TC*, .058 for *NVP+ADV*,

.061 for  $NVP+3TC+ADV$ , and .370 for  $3TC+ADV$ . There were no mutations but 184 reversal (and two other reversals) for the *No drug* regimen. Clearly, none of the five p-values is less than .05. However, when we combine them by Fisher's celebrated technique<sup>5</sup> of summing minus twice the natural logarithms of the p-values and comparing the sum with a chi-square distribution with 10 degrees of freedom, the overall p-value for the null hypothesis of "under-dispersion" comes to .03. For this reason we did not use the Poisson model for numbers of mutations. Instead, our test statistic was nonparametric. Given two candidate drug regimes, it was simply the difference between cumulative numbers of mutations, pooled across isolates. The significance of this difference at each passage was assessed by a permutation test<sup>6</sup>.

### REFERENCES FOR APPENDICES

1. Miller, Jr., R.G.: Simultaneous Statistical Inference. 1981; 2nd Edition: 69-70.
2. Raikov, D: On the decomposition of Gauss and Poisson laws. *Izv. Akad. Nauk USSR* 1938;2 (in Russian).
3. Rice, J.A.: *Mathematical Statistics and Data Analysis*. 1988;434-436.
4. Efron, B. and Tibshirani, R.J.: *An Introduction to the Bootstrap*. 1993;53-56.
5. Fisher, R.A.: *Statistical Methods for Research Workers*. 1963;19th Edition:99-100.
6. Efron B. and Tibshirani, R.J.: *An Introduction to the Bootstrap*. 1993;202-219.