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A Classical Parallel Line Interlot Potency Comparison of Lots 10 and 15 of Rift Valley Fever Vaccine in an Animal Model[1,2]

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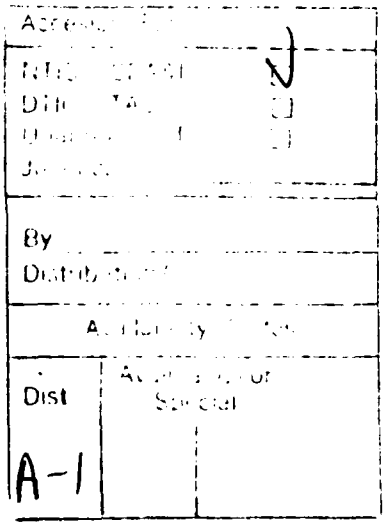
FOOTNOTES

[1]In conducting the research described in this study, the investigators adhered to the Guide for the Care and Use of Laboratory Animals as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[2]The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.



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Twenty lots of an improved formalin-inactivated Rift Valley fever vaccine were prepared in 1978-1979 with well-characterized, diploid fetal rhesus lung cells. Human tests of this new vaccine revealed lot to lot variability in serological response. The plaque-reducing neutralization test was used in a classical parallel line bioassay to measure the differences between the potency of lot 10 and lot 15 in Wistar Furth rats. A twofold difference in potency was detected using minimal resources. The size of the experiment required that the neutralization testing be done in two iterations. This was done by two separate methods, involving sequential and randomized assignment of sera to the neutralization tests. The sequential assignment gave a better estimate of the within-lot dose response. The randomized determination eliminated confounding the between-lot variability with drift in the neutralization test, but at a cost of loss of some of the discernible effect of lot and dose on response.

Introduction

Rift Valley fever (RVF), a viral disease of man and domestic animals, initially found in sub-Saharan Africa, was first described in 1931 (1). The virus causes a serious epizootic disease in sheep and cattle. In humans it typically results in an incapacitating, but self-limited, febrile illness (2). Common complications in humans include ocular disease, encephalitis, and hemorrhagic fever, which occasionally results in death (2). RVF virus has been responsible for frequent laboratory infections (3,4). The major mode of transmission is the mosquito (5). The virus is also infectious by aerosol, a suspected route of infection in both laboratory and epidemic settings (6,7,8).

During 1977 and 1978, epidemic RVF occurred in Egypt. This epidemic was the first recognized outside of sub-Saharan Africa. The attack rate in the unprotected human population was high and serious complications were frequently observed among those stricken (9). Furthermore, devastation of the lamb crop, and significant damage to cattle production occurred.

In 1967, a formalin-inactivated vaccine produced from RVF virus propagated in infected primary monkey kidney cells (Rift Valley Fever Vaccine, inactivated, dried, NDBR-103) was introduced to protect laboratory workers (10,11,12). More than 2000 persons received the vaccine, and, of those tested, more than 95% were found to develop a serological response thought to be associated with protection (11,12,13).

During 1978 and 1979, 20 lots of a new vaccine (Rift Valley Fever Vaccine, inactivated, dried, TSI-GSD-200) was prepared from a clone of the original seed virus propagated in a well-characterized, diploid rhesus monkey cell substrate (14). Human trials of this vaccine revealed marked interlot variation in evoked neutralizing antibody response (15, 16, 17, 18). Three of the four independent trials were flawed by the sequential nature of the entry of subjects into the testing and by the small number of subjects tested.

It was demonstrated that the log-titer neutralizing antibody response in hamsters and humans was a linear function of the log of the dose, and the slope of the resulting line was estimated to be unity (15, 19). Correlation of survival with increasing neutralizing antibody titer has also been shown in rats and hamsters (20,21).

This trial was designed to test whether between-lot variability in the measured serological response to this particular vaccine could be assayed by a classical parallel line bioassay (22).

Materials and methods

Vaccine

Rift Valley Fever Vaccine, Inactivated, Dried, TSI-GSD-200 (lots 10 and 15), was obtained from the manufacturer, The Salk Institute, Government Services Division, Swiftwater, PA., and stored at -20°C. For use, the vaccine was reconstituted with

sterile water for injection as directed in the package insert and used within 2 h of reconstitution. Each vaccine vial was checked for presence of vacuum prior to use.

Animals

Two hundred, male 200-g Wistar-Furth rats (WF/NHsd), an inbred strain, obtained from Harlan Sprague Dawley, Indianapolis, IN, were identified with ear tags and held in a quarantine room for 1 week prior to the experiment. The rats were housed in suspended polycarbonate cages with wire floors. Food, National Institutes of Health open formula rat and mouse ration (NIH-07), obtained from Agway Incorporated, Waverly, NY, was provided ad libitum. Water was provided ad libitum via an automatic system. The animal room was maintained at 22-23°C and 40-50% humiditiy with a 12 h photo period.

Procedures

Each vaccine lot was tested at two doses to minimize the number of animals needed and because the experiment was not designed to test the linearity of response for a given lot, but was optimized to test the difference in response of two lots. Linearity of response was assumed from previous animal studies (17) and from a human trial (15). The two doses, 0.3 ml and 0.6 ml, given subcutaneously on days 0, 7, and 28, were selected based on previous experiments (15,17). Meadors (19) showed previously that differentiation of lot effects upon survival was

increased with a one-dose schedule. However, for this experiment, the three-dose schedule was chosen for two reasons. First, the human vaccination schedule which produced the linear response was the three-dose schedule used for vaccination of laboratory workers (15). Second, the linear dose response in hamsters was observed in a three-dose schedule (19). Lots 10 and 15 were chosen for this test because they were thought to be among the polar lots based on the initial human trials of lots 10 through 18 (17).

The animals were assigned randomly to four groups of 50 animals each. The animals were injected subcutaneously on days 0, 7, and 28 with either 0.3 ml or 0.6 ml of vaccine from either lot 10, run 2; or lot 15, run 1. Animals were observed for 20 min after vaccination for any immediate reaction. They were then observed twice daily for the duration of the study. On day 42, the animals were anesthetised with 13 mg/kg intramuscularly of Xylazine (Haver, Mobay Corporation, Animal Health Division, Shawnee KS), and with 87 mg/kg intramuscularly of Ketamine hydrochloride injection (Fort Dodge Laboratories Inc, Fort Dodge,IA), Blood (2.5 ml) was obtained by cardiac puncture.

Plaque reducing neutralization test

Each serum sample was tested in two plaque-reducing neutralization tests. In the first, sera were assigned to the two iterations of the neutralization test so that sera from rats inoculated with lot 10 were tested in one iteration and sera from

rats inoculated with lot 15 were tested in the other. In the second, sera were assigned randomly to each of the two iterations of the neutralization test. In both cases sera were assayed blindly.

The plaque-reducing neutralization test (PRNT) was similar to that used in the second human trial (16). Each of a series of four-fold serum dilutions was mixed with approximately 80 to 120 plaque-forming units (PFU) virus to yield final dilutions ranging from 1:10 to 1:1280. After incubation at 37°C for 1 h, residual virus was assayed in duplicate VERO cell monolayers in 60-mm wells. The highest dilution of serum reducing the viral titer by approximately 50% was deemed to be the $PRNT_{50}$. The highest dilution of serum reducing the viral titer by approximately 80% was deemed to be the $PRNT_{80}$. Appropriate positive and negative controls were included in each test.

Statistical methods

For purposes of correlation analysis between the two methods of titer determination and for testing of between-lot differences and computation of the geometric mean titers, the $PRNT_{50}$ and the $PRNT_{80}$ were transformed to common logarithms. The between lot and within lot variabilities in $PRNT_{50}$ and $PRNT_{80}$ were quantified by analysis of variance techniques imbedded in

the general linear model of SAS (23). The relative responses to the two lots were estimated by the ratio of the geometric mean titers, and 95% confidence intervals were estimated for the relative potencies by an application of Fieller's theorem (25).

Technical constraints dictated that the neutralization test be done in two iterations. Therefore neutralization testing was done with two different methods to assign the samples. By the first method, sera from each lot were assigned so that each lot was tested in a separate neutralization test. By the second method, sera from each lot were randomly assigned to the two separate neutralization tests. Given that each serum was to be tested twice, one test resulting from sequential assignment of sera to iterations of the neutralization test, and the other from random assignment of sera to iterations of the neutralization test, we decided to test for comparability of the two results. For this comparison of the pair of values of the $PRNT_{50}$ and the separate comparison of the pair of values of the $PRNT_{80}$, the titers tranformed to common logarithms were again used. The paired T test was then applied to test for the null hypothesis of equality, that is, a difference of 0.0 between the two determinations of each titer.

Results

Figure 1 depicts the $PRNT_{50}$ and the $PRNT_{80}$ responses to lot 10, and Figure 2 depicts the $PRNT_{50}$ and the $PRNT_{80}$ responses to lot 15. The geometric mean titers for each combination of lot

and dose are tabulated in Table 1. The null hypotheses of common slope was not rejected for either the randomized or sequential assignment of sera to iterations of the neutralization test. For the sequential assignment of the sera to the iterations of the neutralization test, $p = 0.802$ and $p = 0.922$, respectively, for the $PRNT_{50}$ and $PRNT_{80}$. For the randomized assignment of sera to the iterations of the neutralization test, $p = 0.184$ and $p = 0.102$, respectively, for the $PRNT_{50}$ and $PRNT_{80}$. Therefore, a line of common slope was fitted to both lots for each method of sample partition to allow analysis of the difference in the intercept of the pairs of lines in each partition.

In the case of the sequential assignment of samples to the $PRNT_{50}$ assay, the common slope of the pairs of lines was estimated to be 1.08. In the case of the sequential assignment of samples to the $PRNT_{80}$ assay, the common slope of the pairs of lines was estimated to be 1.18. In the case of the random assignment of samples to the $PRNT_{50}$ assay, the common slope of the pairs of lines was estimated to be 0.47. In the case of the sequential assignment of samples to the $PRNT_{80}$ assay, the common slope of the pairs of lines was estimated to be 0.64.

The intercept of the pair of lines from lots 10 and 15 when tested by the $PRNT_{80}$ assay, after sequential assignment of the sera was found to differ significantly, $p < 0.0001$. In the case of the sequential assignment of sera to the $PRNT_{50}$ assay, the intercept of the pair of lines was also found to differ significantly, $p < 0.0001$.

The intercept of the pair of lines from lots 10 and 15, when tested by the $PRNT_{80}$ assay after random assignment of the sera, was found to differ significantly, $p < 0.004$. In the case of the random assignment of sera to the $PRNT_{50}$ assay, the intercept of the pair of lines was not found to differ significantly, $p < 0.06$.

For the sequential assignment of the samples to the $PRNT_{50}$ assay, the potency difference was estimated to be 2.08-fold, with confidence limits of 1.49-fold to 3.85-fold. For the sequential assignment of the samples to the $PRNT_{80}$ assay, the potency difference was estimated to be 2.22-fold with confidence limits of 1.59-fold to 4.00-fold. For the random assignment of the samples to the $PRNT_{80}$ assay, the potency difference was estimated to be 2.04-fold with confidence limits of 1.23-fold to 12.5-fold.

Application of the paired T test to the difference between $PRNT_{50}$ from the sequential assignment of sera and those from the random assignment of sera resulted in rejection of the null hypothesis of equality at the $p < 0.0001$ level. The difference between the two determinations of the $PRNT_{50}$ was estimated to be 1.62-fold, with 95% confidence limits of 1.38-fold to 1.90- fold. Application of the paired T test to the difference between $PRNT_{80}$ from the sequential assignment of sera and those from the random assignment of sera resulted in rejection of the null hypothesis of equality at the $p < 0.0001$ level. The difference between the two determinations of the $PRNT_{80}$ was estimated to be 2.40-fold, with 95% confidence limits of 2.05-fold to 2.81- fold.

Discussion

Initial clinical trials of lots 1-8 and lots 10-18 of the new RVF vaccine, TSI-GSD-200, were promising (15,17), but both trials revealed significant lot to lot variability in levels of neutralizing antibody resulting from immunization. This experiment was designed to test for an animal correlation to this measured between-lot variability detected in the human trials. Two doses, 0.3 ml and 0.6 ml of each of lots 10 and 15 of vaccine, were tested. Although the only model that can be fitted to the two pairs of points resulting from testing each of the two lots at two different doses is the linear model, fitting this model was reasonable, given the hamster and human response data.

The results of this study indicate that the two lots differed by approximately twofold in their relative potencies, regardless of which method of assignment of sera to neutralization test was utilized. In this rat study, lot 10 was more potent than lot 15. In the initial human study (17), lot 15 was ranked higher that lot 10, but the difference was statistically significant only on day 42. In the initial human study, the lot to lot differences in potency were confounded with drift in the neutralization test and lack of demonstrated comparability of groups of volunteers. Both confounding effects are due to the sequential nature of the recruitment of volunteers against the lots of vaccine. In the ongoing, randomized test of all lots of the vaccine, there were no significant differences

detected among the lots in terms of response. It must be stressed that this ongoing study (18) is in its early phases and, hence, grossly underpowered in terms of detecting even biologically significant differences among the lots. Therefore, we believe that this animal experiment correctly ranks the two lots and will speculate that a human study of sufficient size would detect similar differences in potency.

The initial human study (15) revealed that the dose response of humans to 0.1, 0.3, and 1.0 ml doses of Lot 1 of the vaccine was linear with a slope of unity. A single neutralization test of all the sera involved was used in the estimation of the slope. Unpublished work with hamsters (19), the slope of the dose response was estimated to be approximately unity. Again, all the sera were tested in a single neutralization test. In this experiment, when single lots were tested in single neutralization tests, as was the case in the sequential assignment of lots to iterations of assay, the slope of the dose response curve was again found to be unity. It is our opinion that over practical dose ranges for effective lots of the vaccine, humans, hamsters and rats all demonstrate a dose response slope of unity.

It could be argued that three doses of the vaccine should have been tested in this experiment. We believe that such an experiment would have been wasteful of animal resources. Furthermore, if the response were not linear, as assumed by our analysis, then it would have been highly unlikely that the forward difference would have been found to have the equivalent

of a slope of unity in both cases. This supports our hypothesis that we are indeed dealing with a dose response that is linear in the region we have tested.

The fact that the slope of the dose response differences was not estimated to be unity when the samples were partitioned randomly into two neutralization tests, is the result of the nature of estimation of slope by analysis of variance techniques imbedded within the general linear model technique. As sample variance increases, the estimate of the slope of a line is reduced, so that in the case of a purely random association between a stimulus and a response, the slope of the line will not differ significantly from 0.0. Assigning sera to two separate neutralization tests increased sample variability, did not change the degrees of freedom, and, hence resulted in an estimate of the slope less than unity, as we observed. The increase in sample variance associated with the randomized assignment of sera to the iteration of the neutralization test increased the size of the 95% confidence limits in the estimation of relative potency of the two lots. In the case of the $PRNT_{50}$, the difference ceased to be statistically significant.

The repeated titers differed statistically at a $p < 0.001$ level and the differences were 1.62-fold and 2.40-fold, respectively, for the $PRNT_{50}$ and the $PRNT_{80}$. Because the repeated testing involved a permutation of the data followed by division between two iterations of the neutralization test, confounding was present between any temporal effects, such as

drift in the neutralization test; and an effect resulting from the permutation of the order of the sera prior to division among the two iterations. Serologists, in general, test a single negative and a single positive serum and do not consider a twofold or less difference significant.

This animal model best mimics the situation in human laboratory workers where the serological response is used to determine whether or not a given individual can enter a laboratory in which the virus is in use. Other than the technical constraint imposed by the limits to the size of a PRNT, the model is widely applicable to similar vaccines produced in multilot quantities. This model is efficient in that lot to lot variability is measured with a relatively small number of animals. We believe that, in similar circumstances where human response is measured by a serological assay, lot to lot potency of multilot vaccines should be tested in this manner before establishing the human response. Such an animal model would also be of practical value in comparing any new putative vaccine to the standard vaccine.

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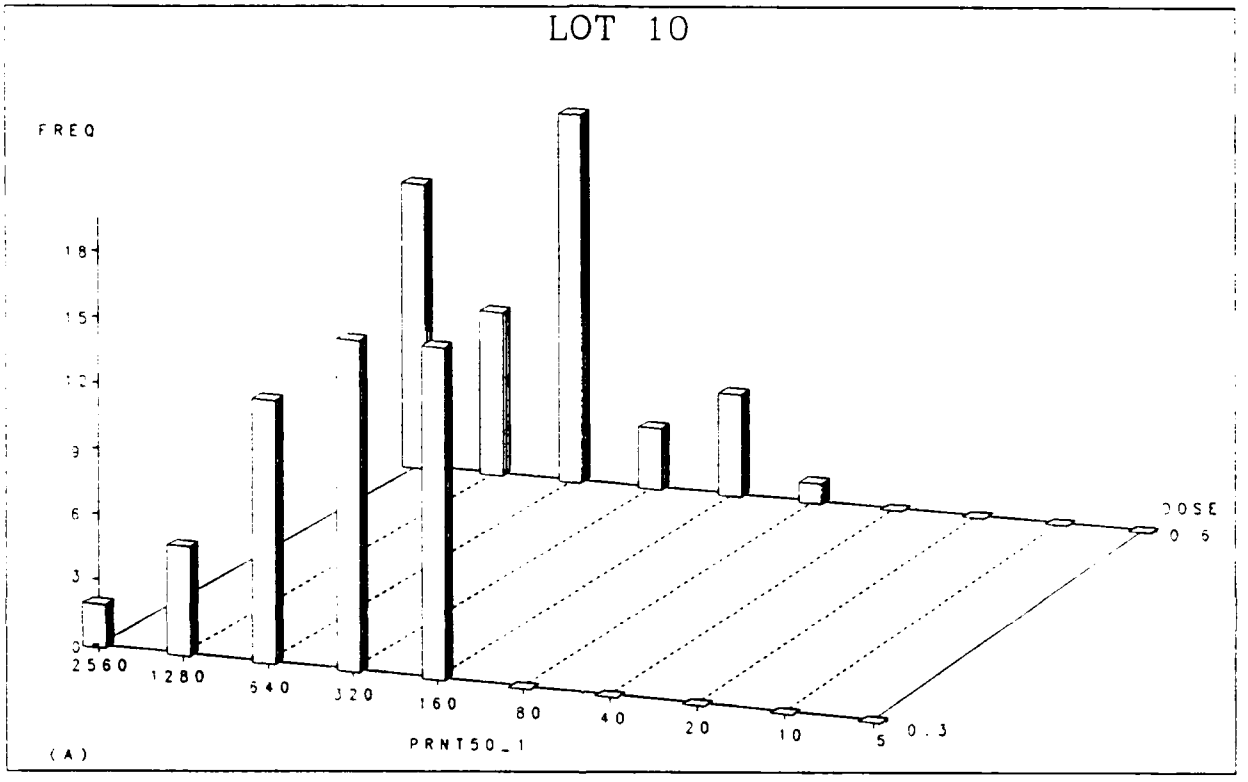
Figure 1. (A) The $PRNT_{50}$ response of Wistar – Furth rats to RVFV vaccine, Lot 10. (B) The $PRNT_{80}$ response of Wistar – Furth rats to RVFV vaccine, Lot 10. The rats were vaccinated with 0.3 ml or 0.6 ml of vaccine subcutaneously on days 0, 7, and 28. Serum was obtained on day 42.



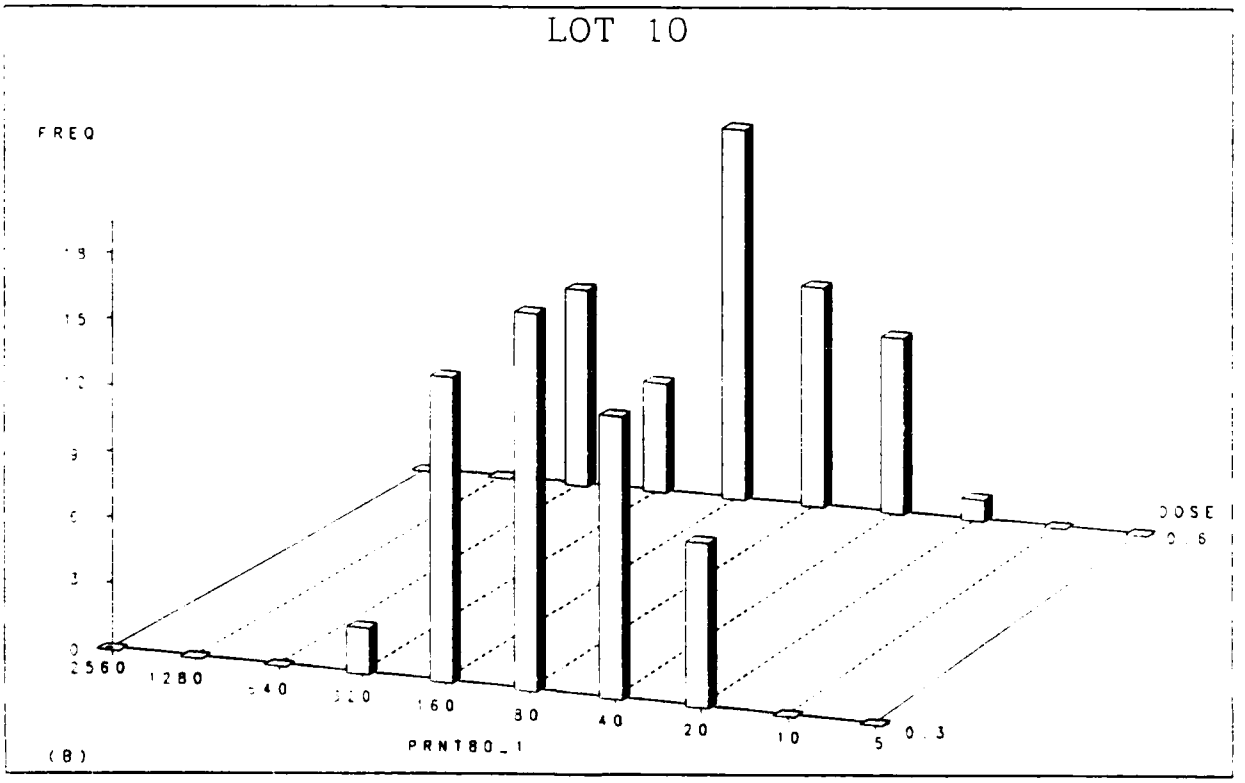
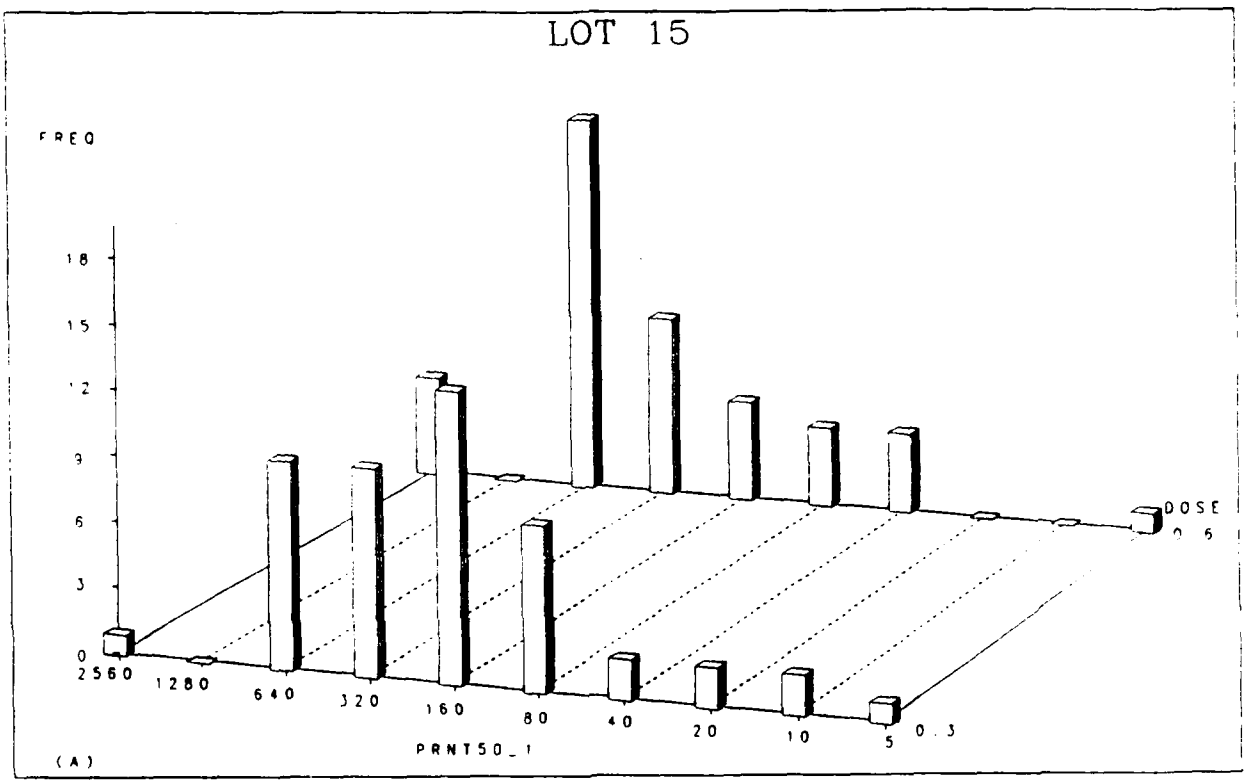


Figure 2. (A) The $PRNT_{50}$ response of Wistar-Furth rats to RVFV vaccine, Lot 15. (B) The $PRNT_{80}$ response of Wistar-Furth rats to RVFV vaccine, Lot 15. The rats were vaccinated with 0.3 ml or 0.6 ml of vaccine subcutaneously on days 0, 7, and 28. Serum was obtained on day 42.



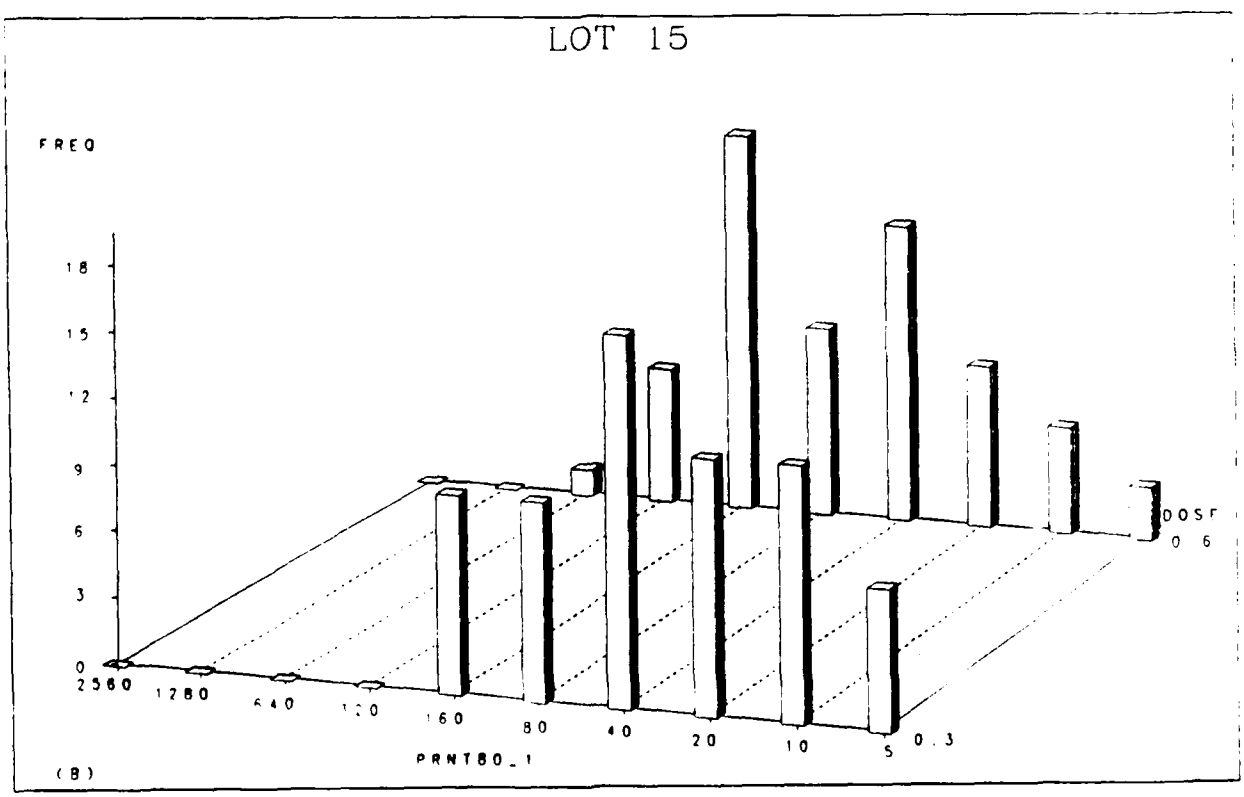


Table 1 Response of WF Rats to RVFV Vaccine (TSI-GSD 200)*

Lot	Dose (ML)	Geometric Mean Titers (95% Confidence Limits)	
		$PRNT_{50}$	$PRNT_{80}$
10	0.3	411 (320 -527)	71 (57 - 87)
	0.6	892 (678 - 1174)	147 (113 - 191)
15	0.3	167 (118 - 235)	30 (22 - 40)
	0.6	393 (265 - 584)	64 (46 - 89)

*Response of Wistar-Furth rats to RVFV vaccine. Male rats weighing 200 grams were vaccinated subcutaneously on days 0, 7, and 28. Serum was obtained on day 42 for determination of the plaque reducing neutralization titer (PRNT).

Running head: Rift Valley fever vaccine