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A STUDY OF VARIATION IN THE NUMBER OF POCK LESIONS FORMED ON THE CHORIOALLANTOIC MEMBRANES OF CHICK EMBRYOS BY TITRATING NATIONAL REFERENCE SMALLPOX VACCINE VIRUS.

C. L. SEHGAL[1], D. K. MURTY[2], P. SINGHA[3], J. B. SHRIVASTAV[4], AND N.G.S. RAGHAVAN[5].

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INTRODUCTION.

LITTLE published information is available regarding the variation in the number of pock lesions formed on the chorio-allantoic membrane of developing chick embryos as a result of titration of smallpox vaccine virus for determining the potency of the vaccines in this experimental host using the same vaccinia virus material. This has a bearing on the biological standardization of smallpox vaccine.

Fenner and McIntyre (1956), while conducting the infectivity titrations of myxoma virus in developing chick embryos, found a wide scatter in the pocks produced on the chorio-allantoic membrane. They attributed it to the variation in the susceptibility of egg membranes. Their figures showed co-efficients of variation varying from 28 to 110 per cent with a majority lying above 60 per cent in a series of 36 titrations of the same material carried out by them. Kaplan and Belyavin (1957) carried out 115 titrations of vaccinia virus preparations in chorio-allantoic membrane of chick embryos (CAM) and found that the count variances 'Wandered' so extensively that no valid estimate of the overall co-efficient of variation was possible.

The authors while carrying out tests of batches of smallpox vaccine either indigenously manufactured or imported, noted a variation in the number of pock lesions formed on the CAM of developing chick embryos using the same 'Batch' of vaccinia virus. This prompted them to study in detail the variation in the number of pocks produced on the membrane of different eggs with the same vaccinia virus material using the National Reference Smallpox Vaccine prepared at the State Vaccine Institute, Patwadangar (India) as the standard.

1. Assistant Director, Microbiology Division, National Institute of Communicable Diseases, 22-Alipore Road, Delhi-6.
2. Formerly Deputy Director, Microbiology Division, National Institute of Communicable Diseases, 22-Alipore Road, Delhi-6. *Present Address*: U. P. College of Veterinary Sciences and Animal Husbandry, Mathura, U.P.
3. Assistant Director, Statistics, National Institute of Communicable Diseases,22-Alipore Road, Delhi-6.
4. *At Present*: Additional Director General of Health Services. He was Director, N.I.C.D., Delhi, up to April, 1967.
5. Director, National Institute of Communicable Diseases, Delhi, since May, 1967.

Materials and methods.

A number of ampoules under two batch numbers F.D. 25 II/66 and S.D. 25 I/66 each containing 25 doses of freeze-dried smallpox vaccine were obtained from the State Vaccine Institute, Patwadangar, India. This vaccine was tested each time as a National Reference Vaccine Control along with the other batches of the vaccine for determining the potency and total bacterial count.

For the current studies the contents of four of these ampoules were reconstituted in an appropriate volume of McLivaine's citric acid/disodium phosphate buffer pH 7·2 to have 1 : 10 (10^{-1}) dilution of the vaccinia virus. From this ten-fold serial dilutions ranging from 10^{-2} to 10^{-6} were made using the same diluent. These dilutions were stored in the ice chamber of the refrigerator prior to inoculation on the dropped chorio-allantoic membrane of the chick embryos.

Fresh fertilized hen's eggs from disease-free flock were purchased from the Government Poultry Farm, Delhi, in order to have the same source of supply throughout. On their receipt, the eggs were cleaned with water to remove faecal material and dust, wiped with spirit or 2 per cent Savlon solution and then kept in a commerical egg incubator at 38°C. for 12 days. The eggs were candled and those having active embryos and normal air sacs were used.

The technique of inoculation on the chorio-allantois of 12-day old chick embryos described by Westwood *et al.* (1957) was followed. Inoculation was usually carried out after one-and-a-half to three hours time after dropping the membranes and during this period the eggs were kept in the bacteriological incubator at 36°C. 0·1 ml. of inoculum comprising of 10^{-5} and 10^{-6} dilutions of the virus was introduced into the dropped chorio-allantois and five to six embryonated eggs were employed for each dilution. The eggs were then rocked gently to spread the inoculum on the chorio-allantoic membrane. The holes on the shell were then sealed with cellophane tape. The eggs were then incubated in the incubator at 36°C. for about 48 hours in a horizontal position with cellophane seal upper-most.

After 48 hours incubation, the shell of each egg was cut longitudinally with scissors at two different places. The embryos, yolk sac and the albumin sac were then removed and discarded. The CAM from dead embryos or showing haemorrhages were also discarded.

From the others, chorio-allantoic membrane around the site of inoculation was removed, washed in water and spread in the Petri dish which was placed on a black board. The pock lesions were then counted and the average pock count calculated. The titre of the virus was then determined for 1 ml. of undiluted vaccine.

Results.

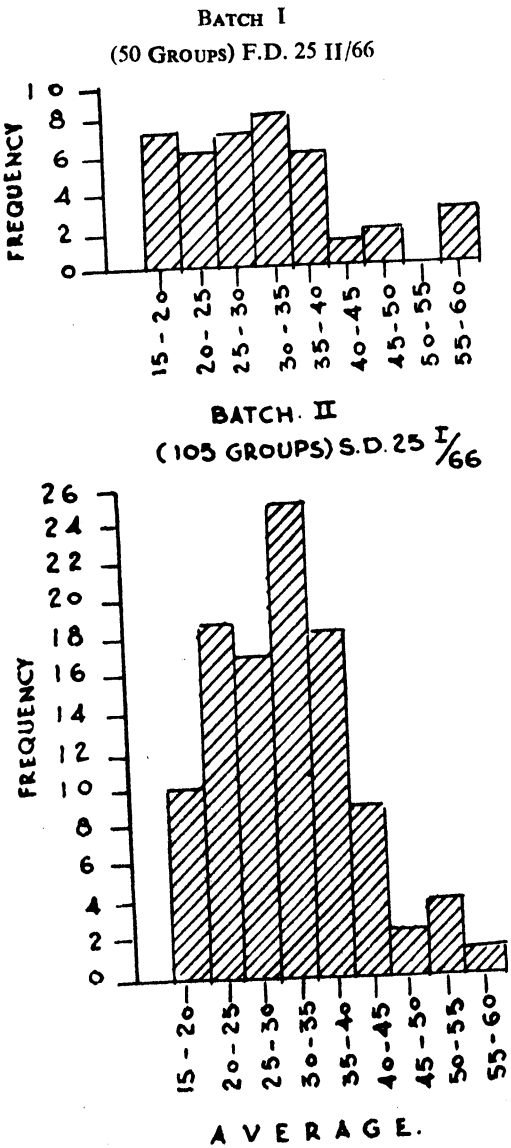
The series of observations recorded were in fact from two batches of lyophilized smallpox vaccine having different manufacturing batch numbers. The analysis of the data was made separately for each of these batches. The first batch of vaccine (F.D. 25

II/66) was tested during the period from 1-9-1966 to 19-9-1967, while the testing of second batch of vaccine (S.D. 25 I/66) started from 25-9-1967 and was completed on 24-9-1968.

The first batch was tested for 50 times, each time taking 5-6 embryonated hen's eggs 12-day old in a group. The second batch was tested for 105 times taking the same number of eggs.

GRAPH 1.

Histograms of average pock count from groups of 5-6 eggs.

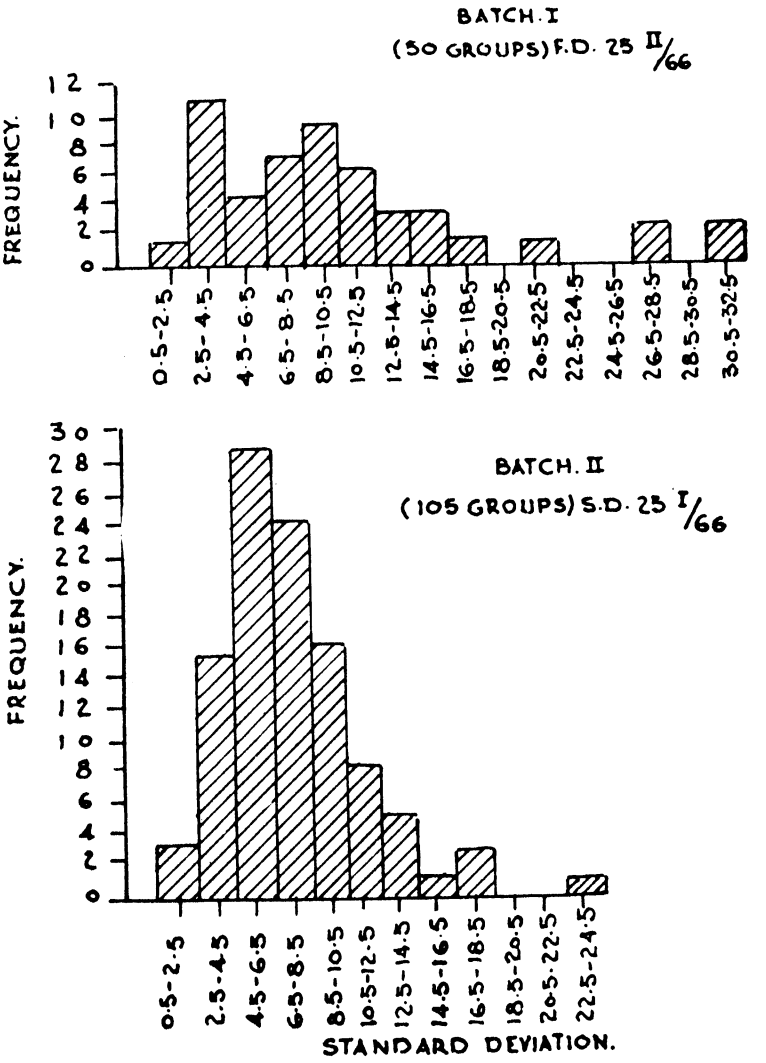


The overall mean value and the standard deviation of the pock count for all the individual readings of the eggs were 27·1 and 5·3 respectively for the first batch and 31·3 and 3·6 respectively for the second batch. The co-efficient of variation of the former was 19·6 per cent and for the latter 11·5 per cent. It is, thus, evident that the first batch gave lower mean value associated with higher variation as compared to the second batch.

The mean values and standard deviation of each group of 5-6 eggs tested together showed wide variation in both the batches. The mean values of the groups of 5-6 eggs varied from 10 to 58 in the first batch and 12·6 to 58·0 in the second batch, and the

GRAPH 2.

Histograms of standard deviation of pock count from groups of 5-6 eggs.

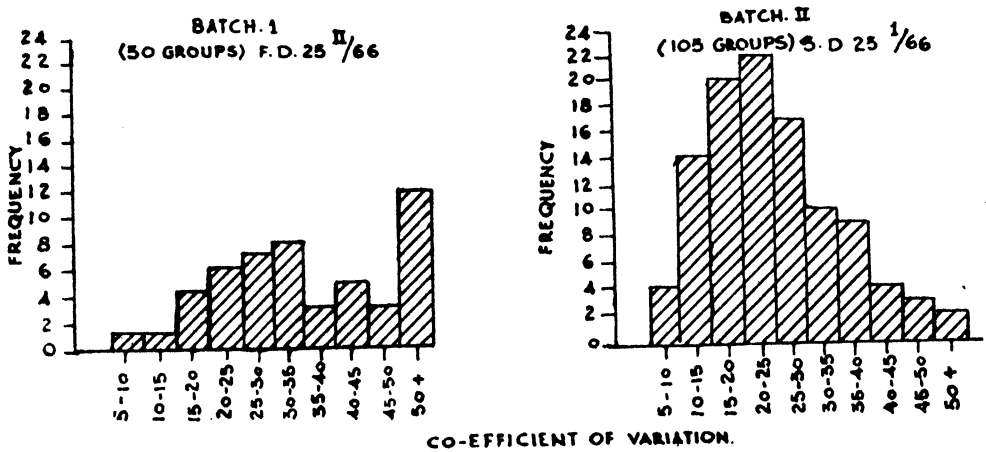


standard deviation between 2·07 and 31·31 in the first batch and 2·23 to 23·69 in the second batch. In the first batch, however, 42 per cent of the groups showed their mean value between 25 and 40. Within this range lay the mean value of about 57 per cent of groups of the second batch. The co-efficient of variation of the first batch fluctuated from 6·38 per cent to 93·97 per cent though 76 per cent of the groups gave rise to co-efficient of variation less than 50 per cent. Second batch similarly showed fluctuation of co-efficient of variation between 9·1 per cent and 76·7 per cent though 98 per cent of the groups had co-efficient of variation less than 50 per cent.

The distributions of mean, standard deviation and co-efficient of variation between the groups within each batch are shown in the Graphs 1, 2 and 3.

GRAPH 3.

Histograms of coefficient of variation of pock count from groups of 5-6 eggs.



The group results of each batch were analysed statistically in order to determine if there was any cognizable significant variation from group to group in the same batch. Analysis of variance technique was employed to test the significance of the difference of the group means for each batch. The results of the analysis are set out in Tables I and II.

TABLE I.

Analysis of variance.

Pock count of first batch tested in groups of 5-6 eggs.

Source of variation.	Degree of freedom.	Sum of squares.	Mean squares.	F.	P.
Between the means of the groups	49	41673·26	850·47	5·67	<0·1 per cent
Within groups	209	31446·57	150·46		
Total	258	73119·83			

TABLE II.

Analysis of variance.

Pock count of second batch tested in groups of 5-6 eggs.

Source of variation	Degree of freedom.	Sum of squaress.	Mean square.	F.	P.
Between the means of the groups	104	41771·25	401·64	5·75	<0·1 per cent
Within groups	439	30366·49	69·85		
Total	543	72137·74			

For the group means, the observed variance ratio is associated with the probability less than one per cent signifying that the difference between the group means of each of the batches cannot be explained away as chance variation or in other words there seems to be a significant difference between group means.

This variation may probably be caused by the degree or susceptibility of individual chick embryo, fluctuation in temperature of incubator owing to fluctuation in the electric current and high ambient temperature during summer months, non-uniformity of graduated pipettes for making viral dilutions, etc.

It was of interest to examine further whether the two batches differed in respect of the variation of potency of vaccine. In the analysis of variance the total variation of pock count of individual eggs was separated into two components (1) due to variation within group, and (2) variation between group. All these types of variations, i.e. (*i*) 'Total variation', (*ii*) 'Between variation', (*iii*) 'Within variation', were compared between the two batches by means of two sided F-test. The values of F for 'Total variation', 'Between variation' and 'Within variation' were 2·13 (258 and 543 D.F.), 2·12 (with 104 and 49 D.F.) and 2·15 (209 and 439 D.F.) respectively. Each of these F's is associated with probability less than one per cent showing that variation between the batches either in 'Total variation', or 'Between group variation' or 'Within group variation' cannot be explained by chance alone.

DISCUSSION.

The results of the statistical analysis point to fact that stability in the pock count is difficult to attain inasmuch as not only the individual eggs vary when tested in group of 5-6 eggs from the same batch, the group means also vary significantly. Added to this variation is imposed another significant variation due to varying batches supplied by the production centres.

It may be explained that significant 'Within group variation' points to the intrinsic heterogeniety of either susceptibility of the eggs or other factors which determine the response in chick embryo. Significant 'Between variation' similarly shows the influence of extraneous factors in the experimental set up which are possibly

uncontrollable. Batch to batch variation may be due to lack of manufacturing uniformity.

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