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Overlap and the errors of plaque counting

I. The overlap biases of observed counts and their correction

By D. W. HOWES

Viral Products Section, National Biological Standards Laboratory, Commonwealth Department of Health, Melbourne, Victoria, Australia

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INTRODUCTION

When viruses multiply in layers of host cells, the number of plaques counted will often underestimate the number of plaque-forming units, owing to overlapping. This 'overlap bias' is also present in other counting techniques and, because it may rival the random error in magnitude, is of practical importance.

In 1949 Irwin, Armitage & Davies developed an approximate mathematical model of overlapping which might be used to correct dust-particle or bacterial-colony counts. This model was improved by Armitage (1949) and shown to be effective in practice. Further results are given by Roach (1968).

However, the assumptions of particle or colony counting represented by this model differ from those of plaque counting in one important respect. Whereas in particle counting each clump is counted as one, in plaque counting the observer attempts to resolve and count all members of the large and irregular areas of lysis which are produced by plaque 'clumps'. A mathematical model representing such a counting technique must therefore make allowance for factors influencing resolution. This renders the problem both more complex and less tractable due to the subjective nature of overlap resolution.

The present paper describes an attempt to provide an acceptable solution.

THEORETICAL

(1) *The model*

In the simple model of Irwin *et al.* the concentration of circular particles of equal size, distributed at random on a plate, is measured by the quantity ψ, which is the ratio of the sum of areas of the particles to the area of the plate. Considering only circular plates, and in the present notation,

$$\psi = \frac{Nd^2}{D^2}, \tag{1}$$

where N = number of particles, or true count, d = particle (or plaque) diameter, D = plate diameter. The formula relating the true count N and the observed count C is

$$C = \frac{N(1-e^{-4\psi})}{4\psi}. \tag{2}$$

This may be written more conveniently as

$$C = \frac{1}{K}(1 - e^{-KN}), \tag{3}$$

where

$$K = \frac{4d^2}{D^2}$$

and is called the assay constant.

Dulbecco & Vogt (1954) briefly considered the problem of overlapping in plaque counting and visualized this in terms of the critical distance (g) between plaque centres below which overlapping plaque pairs are not resolved. In effect the assay constant in their model is

$$K = \frac{4g^2}{D^2}. \tag{4}$$

A similar approach was adopted by Lidwell in appendix V of Bourdillon, Lidwell & Lovelock (1948). However, the practical value of the above model and that proposed by Cooper (1961), which is essentially the same, is limited, as the various factors which determine the critical distance have never been defined or separated.

One factor is certainly the average size of plaques, while others include plaque morphology, the variation of plaque size, etc., all of which influence the capacity of the observer to resolve overlapping plaques. The combined effect of these other factors may be represented by a single 'resolution factor', P, defined as the ratio, g/d, of the critical overlap distance to plaque diameter d. This leads to

$$K = \frac{4P^2d^2}{D^2}. \tag{5}$$

P will have values ranging from 0 for plaques which are always resolved no matter how close their centres, to 1 for plaques which become unresolvable as soon as they touch. The latter extreme corresponds to the 'clump' counting of particles considered by Irwin *et al.*

The replacement of g by its factors P and d allows evaluation of the effect which changes in plaque morphology will have upon an observer's capacity to resolve overlapping plaques. It also paves the way to practical application, for, once the value of P for a standardized assay system has been determined, the assay constant, K, can be obtained by measuring a number of plaques and performing a simple computation.

In practice equation (3) has been used in the form

$$N = -\frac{1}{K}\log_e (1 - KC). \tag{6}$$

The correction factor, the ratio N/C, is specified by KC. Hence a single table of correction factors for a range of values of KC will suffice for all assay systems. This has been prepared and will be presented elsewhere. (Howes & Fazekas de St Groth, in preparation).

(2) *The estimate of the resolution factor P*

The value of P may be estimated by measuring pairs of overlapping plaques, of approximately the same size, which have been barely resolved in the course of routine plaque counting. By definition, the smallest observed value for the ratio of the distance between their centres to their diameter should approximate the value of P.

An alternative method is to calculate the value of P from observed decreases in plaque counts which accompany a known increase in plaque size, i.e. are due to overlapping. As is shown in the appendix the relationship is

$$P^2 = \frac{D^2(C_1 - C_2)}{2(C_2^2 d_2^2 - C_1^2 d_1^2)}, \tag{7a}$$

where the subscripts 1 and 2 designate observed counts and mean plaque diameters for the first and second counting times respectively.

Estimation of the value of P^2 by means of equation (7*a*) requires the processing of considerable volumes of counting data, and although the mean counts observed on groups of replicate cultures may be used, this would often require that special experiments be carried out. This disadvantage is overcome if an estimate of P^2 is obtained from each culture, and all estimates are then weighted and pooled to give a combined estimate. Data provided by cultures drawn from routine assays may then be used.

The error of the initial count C_1 vanishes in this method of analysis, and the major component of the error of P^2 becomes the error in estimating the number of plaques missing from a particular plate at the second counting, $(C_1 - C_2)$.

To facilitate calculations, a further simplification of equation (7*a*) can be made,

$$P^2 = \frac{D^2(C_1 - C_2)}{2C_1^2(d_2^2 - d_1^2)}. \tag{7b}$$

Although a small systematic error results, which increases the value of P^2 slightly, this tends to compensate for the neglect of more complex forms of overlapping.

Regarding $C_1 - C_2$ as approximately a Poisson variate would lead to a weight, C_1^2, for each value of $C_1 - C_2/C_1^2$. When equation (7*b*) is used, as in these studies, the following convenient relationship is then obtained:

$$\text{estimator of } P^2 = \frac{D^2}{2(d_2^2 - d_1^2)} \left(\frac{\Sigma(C_1 - C_2)}{\Sigma C_1^2} \right). \tag{8}$$

(3) *The inadequacy of simple mathematical models*

Were a complete model of the effects of plaque overlap used, the estimates of P obtained by the two methods should be the same. However, it was found that the estimate of P derived from counting data in the present system was approximately 0·3, whereas that obtained by the inspection and measurement of barely resolved plaque pairs was 0·22 or less. Thus actually more plaques are obscured by overlap than can be explained by the simple model. Correction of this deficiency would

require either a more complex theoretical model or an empirical modification of the simple model. Of these alternatives the latter appeared to have more practical merit.

(4) *The empirical method*

The empirical adjustment of the simple model is achieved by accepting the higher value of P derived from counting data according to equations (7) or (8). Equation (6) then predicts overlap bias errors with acceptable accuracy over a substantial part of the possible counting range. Such values of P are, of course, not covered by its definition, and P^2 is replaced in the relevant equations by the empirical resolution factor 'R'. The estimator of K is then

$$\hat{K} = \frac{4Rd^2}{D^2}. \tag{9}$$

(5) *Allowance for variation in plaque size*

The plaques present on a culture at a given time always show some variation in size, and this will influence the magnitude of the overlap bias to some extent. Following Armitage (1949), d^2 was therefore replaced in equation (5) by

$$(d^2 + 0{\cdot}5s_d^2),$$

where s_d^2 is the variance of the distribution of plaque diameters. The contribution of the variance is, however, relatively small (usually less than 5% of d^2). This refinement has therefore only a minor effect and may be safely omitted in less exacting routine assays.

MATERIALS AND METHODS

Cultures

The cynomolgus monkey kidney cultures used in these experiments were prepared as described by Thayer (1965).

Media

Phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) was used to wash cultures, and with the addition of 0·1% calf serum, which had been heated to 68° C. for 1 hr. to destroy virus inhibitors, was used to prepare virus dilutions. The overlay medium, developed by Mr E. H. Ridge of this laboratory, had the following composition:

Two-fold nutrient solution	ml.
Tenfold concentrated Earle's salt solution	10
2·8% $NaHCO_3$	5
2 M Tris, pH 7·3	1
2 M-$MgCl_2$	1·25
Calf serum, heated at 68° C. for 1 hr.	4
2% yeast extract	5
Neutral red, 1/1000 solution in distilled water	2·5
Distilled water to	50

Penicillin and streptomycin were included in the above solution at 100 units and 100 μg./ml. respectively.

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Prior to use, the nutrient solution was saturated with CO_2, raised to about 44° C., and mixed with an equal volume of 2 % agar at the same temperature.

Virus strains

Two strains of poliovirus were used in four experiments: the attenuated type 1, LSc-2ab strain, and the virulent type 3, Saukett strain.

Inoculation and incubation

Cultures were rinsed twice with PBS, and a 0·5 ml. inoculum was then carefully distributed over each cell monolayer. One hour at room temperature, on a level surface, was allowed for adsorption. After adding 10 ml. of overlay, cultures were incubated at 35° or 37° C. in a humidified atmosphere of 5 % CO_2–air mixture. The incubators had been extensively modified to give better control of temperature and gaseous environment than is usually possible with the bacteriological incubators commonly employed for this purpose.

Plaque counting

In each experiment, plaques were counted on three occasions during a period of from 2–3 days. The plaques present at the first counting time ('original' plaques) were marked with a felt-tip pen containing an acetone soluble dye. Immediately prior to recounting, at the second or third counting times, the relatively few new plaques were marked with indian ink, and the markings of the original plaques were selectively removed with acetone. The original plaques were then recounted again using the felt-tip pen.

Measurement of plaque diameters

In the four experiments the diameters of 194, 107, 107, and 94 plaques, respectively, were measured at each counting time. To do this, cultures inoculated with the highest dilutions of virus were used so that the number of plaques was usually less than ten. All plaques whose centres fell within a scribed concentric circle of 6·1 cm. diameter (internal diameter of the culture plate being 7·2 cm.) were measured on the three occasions.

Experimental design

Apart from the inherent random sampling errors, the random component of counting uncertainty, and the overlap bias, plaque counts may vary owing to non-uniformity of cell cultures, variation in the environment of cultures during incubation, and counting bias on the part of the operator. The following steps were taken to avoid or nullify these sources of error.

(1) All experimental cultures were numbered and then randomized with respect to the virus inoculum by means of sets of random numbers.

(2) Particular care was taken to ensure that all cultures received the same volume of inoculum, that this was distributed evenly, and that variation in the adsorption period was kept within narrow limits.

(3) After overlaying, cultures were placed on numbered trays in numerical order

and the trays were then placed in an incubator in a predetermined order, a procedure which randomized cultures with respect to their position in the incubator.

(4) Cultures were identified only by their assigned numbers and were counted without reference to the records of the inocula they had received.

(5) Plaque overlap also reduces the variance of plaque-count distributions, an effect which is considered in the accompanying paper (Howes & Fazekas de St Groth, 1969). Bias, which could arise if the observer knows or might successfully guess the inoculum which a culture has received, will mimic this effect of overlapping. To avoid this, dilution steps were closely spaced to make it difficult for the observer to guess the inoculum which a culture had received. In the first experiment a series of 1·6-fold dilutions was used but, although apparently successful, even better safeguards were introduced in the remaining three experiments. In these, two series of twofold dilutions of the same virus suspension were used as inocula for sets of replicate cultures, the lowest dilution of the second series being a 9/10 dilution of the lowest dilution of the first. Thus pairs of extensively overlapping distributions were provided at four more widely separated levels of virus concentration. As the observer can do no more than guess from which distribution a culture is derived, any unconscious tendency to bias will be as often as not in the wrong direction, and may be expected to increase rather than decrease the variance of either or both distributions.

ANALYSIS OF COUNTING DATA

Correction of counts

Mean observed counts were corrected by use of the table of correction factors mentioned above. The statistical analysis of ungrouped data was done by a computer, the programme being based on equation (6).

Identification of the limit to the counting range and the estimation of the resolution factor

The value of the empirical resolution factor R is estimated by means of equation (8), using only counting data which fall within the range of KC values for which the correction procedures are valid. However, the limit value of KC cannot be defined until the value of R is known, and both must therefore be estimated simultaneously using an iterative procedure.

Arbitrary values for both are adopted at the start of the first cycle of computations and the estimates provided by this cycle become the starting-point for the next cycle of computations. The recomputation of R during each cycle is simply achieved by adding or subtracting a few values of $(C_1 - C_2)$ and C_1^2 to or from their sums in equation (8). The limit value of KC is determined by testing correction procedures as described below.

The testing of the model and of correction procedures

Using mean counts for groups of replicate cultures

A widely used method analyses data obtained at the one counting time, the corrected counts at one inoculum level being compared with those at other levels. The criterion of success is the conversion of a curvilinear relationship between observed counts and relative virus concentration into a linear relationship for corrected counts. This approach has the disadvantage that it involves comparisons of independent samples, and conformity to or departures from linearity must be assessed against a background of substantial random sampling error.

The alternative method, designed to avoid this problem, is therefore more efficient and was used in this study. In this method, plaque counts observed on one group of replicate cultures, at one time, are compared with counts on the same cultures after further periods of incubation during which plaques increase in size.

Although successive observed counts of the plaques on a culture are expected to decrease as plaque size increases, each provides an estimate of the one true count after correction by the procedures under test. If these procedures are adequate, the ratios of the estimates to one another will fluctuate about the value of 1. In this form of analysis comparisons are made within groups, and the random error of the total true count for the group, so important in the first method, becomes irrelevant.

Using ungrouped cultures

The use of mean counts has implications which may be of little practical importance but must be considered in a detailed analysis of plaque counting. The mean counts are themselves subject to fluctuation due to departures of the observed frequencies from their theoretical distribution, and this scatter contributes to the variance of mean count ratios about the expected value of 1. However, by determining the individual ratios for each culture this effect is avoided. This procedure also permits other factors which cause fluctuation in the ratios of corrected counts to be assessed, and will be discussed with experimental results.

EXPERIMENTAL RESULTS

The effectiveness of correction procedures and the setting of counting limits

Data from groups of replicate cultures

Results of the four experiments are summarized in Fig. 1, and show that for the Saukett strain of virus ratios of corrected mean counts fluctuate about the expected value of 1, for all values of $K\bar{C} \leqslant 0{\cdot}2$. This value sets the limit to the acceptable counting range.

Ratios for the LSc-2ab strain of virus showed greater inherent variation, and although they fluctuated about the value of 1 for low values of $K\bar{C}$, the point at which corrections become inadequate could not be readily recognized.

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Analysis of data from individual cultures

Using the same data, each of the three observed counts of each culture was first corrected by means of equation (6), and then the three ratios of corrected counts, N_2/N_1, N_3/N_2 and N_3/N_1 were calculated. The regression of these ratios on KC was computed, taking progressively widening ranges of the data. For each range of values of KC the following statistics were determined: the slope and intercept of the regression line, together with their errors; the mean ratio of corrected counts for each increment and its error; and the regression and error variances. All data provided by a culture were included in a given range if the value of K_3C_3 fell below the specified upper limit—all data provided by a culture were excluded if this condition was not met.

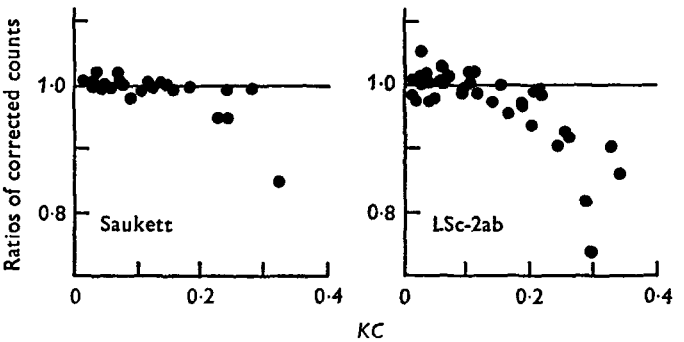

Fig. 1. Ratios of corrected mean plaque counts for the possible counting range. Plaques present at the first counting time on groups of from 13–30 replicate cultures were re-counted at two later times. Mean counts were corrected, and ratios of the second to first, and third to second corrected counts were determined. These fluctuate about the expected value of 1 in lower regions of the possible counting range.

The significance of departures of incremental mean ratios from the expected value of 1 were assessed in a t test. The significance of departures from linearity was assessed by Fisher's F-test.

The computer programme for these operations was written by Dr S. Fazekas de St Groth of the Division of Animal Genetics, C.S.I.R.O.

The results of these analyses are given for the Saukett strain of virus in Table 1, and for the LSc-2ab strain of virus in Table 2, and are discussed under several headings.

Limit to the acceptable counting range

The corrections are adequate as long as (1) the slope of the regression line does not significantly differ from zero, and (2) the regression variance does not show significant trend.

For the LSc-2ab strain of virus (Table 2) significant changes appeared in Expt. 3 as the upper limit to the range of values of KC was increased from 0·18 to 0·20, and in Expt. 2 during the increment from 0·20 to 0·22. However, in the latter experiment a significant change was barely avoided in the increment from 0·18 to 0·20. The limit to the acceptable counting range was therefore set as 0·18 for both experiments.

Table 1. *Statistical analysis of plaque-counting data: Saukett strain of poliovirus*

Range of values of KC	No. of observations in range		Statistics of regressions (ratios, N_2/N_1, etc. $= a+bKC$)						Incremental mean ratios of corrected counts (N_2/N_1, etc.)	
			Slope (b)		Error variance $\times 10^3$		Variance ratio† $F(1, n-2)$			
	Expt. 1	Expt. 4	Expt. 1	Expt. 4	Expt. 1	Expt. 4	Expt. 1	Expt. 4	Expt. 1	Expt. 4
0–0·10	147	87	+0·135	+0·388	1·14	2·11	0·82	3·68	1·001	1·011
–0·12	183	99	+0·146	+0·401*	1·40	1·92	1·80	7·47*	1·007	1·038
–0·14	219	117	+0·094	+0·089	1·34	2·03	1·43	0·61	1·002	0·988
–0·16	255	144	+0·056	−0·032	1·46	2·08	0·78	0·15	1·004	0·987
–0·18	276	165	+0·093	+0·047	1·48	1·96	2·83	0·47	1·018	1·018
–0·20	300	186	+0·033	+0·022	1·64	2·03	0·42	0·14	0·991	1·006
–0·22	330	189	−0·004	+0·022	1·70	2·00	0·01	0·15	0·996	0·999
–0·24	363	189	−0·032	.	1·81	.	0·71	.	0·991	.
–0·26	405	195	−0·150*	+0·026	2·11	1·96	19·44*	0·25	0·958	1·008
–0·28	420	216	−0·147*	−0·046	2·06	2·15	21·53*	1·11	0·981	0·986
–0·30	441	240	−0·276*	+0·017	2·51	2·22	75·80*	0·21	0·871*	1·017
–0·32	474	258	−0·346*	+0·039	2·83	2·19	137·1*	1·39	0·904	1·018
–0·34	486	270	−0·360*	+0·037	2·83	2·13	162·9*	1·51	0·909	1·007
–0·36	498	273	−0·376*	+0·045	2·88	2·13	193·0*	2·39	0·893	1·057*
–0·38	507	.	−0·363*	.	2·93	.	190·8*	.	0·954	.

* Significant value ($P < 0·05$).

† Ratio $\frac{\text{regression variance}}{\text{error variance}}$.

Table 2. *Statistical analysis of plaque-counting data: LSc-2ab strain of poliovirus*

Range of values of KC	Number of observations		Statistics of regressions (ratio, N_2/N_1, etc. $= a+bKC$)						Incremental mean ratios of corrected counts	
			Slope (b)		Error variance $+10^3$		Variance ratio† $F\ (1, n-2)$			
	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3
0–0·10	177	177	+0·142	−0·159	2·78	1·84	0·71	1·26	1·001	1·001
–0·12	204	138	+0·342*	−0·209	2·57	2·98	7·61*	1·99	1·033	0·978
–0·14	231	165	+0·075	−0·050	2·82	2·81	0·54	0·20	0·979	1·012
–0·16	249	180	+0·082	−0·109	2·81	2·75	0·89	1·27	1·013	0·990
–0·18	267	204	+0·011	−0·080	2·84	2·59	0·02	1·10	0·987	1·002
–0·20	285	210	−0·130	−0·188*	2·92	2·79	3·86	6·19*	0·953	0·914
–0·22	297	231	−0·137*	−0·190*	2·88	2·68	5·36*	9·44*	0·986	0·980
–0·24	306	258	−0·123*	−0·265*	2·90	3·49	5·00*	20·44*	0·998	0·961
–0·26	315	276	−0·154*	−0·231*	3·01	3·65	9·05*	18·73*	0·956	0·995
–0·28	342	288	−0·361*	−0·251*	3·87	4·01	59·38*	23·31*	0·894	0·956
–0·30	357	294	−0·462*	−0·238*	4·49	3·94	103·9*	23·37*	0·853	0·984
–0·32	390	300	−0·613*	−0·222*	5·19	4·14	228·5*	21·12*	0·826	0·957
–0·34	402	.	−0·613*	.	5·16	.	256·6*	.	0·876	.

* Significant value ($P < 0{\cdot}05$).

† Ratio $\dfrac{\text{regression variance}}{\text{error variance}}$.

For the Saukett strain of virus (Table 1) the limit was identified as $KC = 0{\cdot}24$ in Expt. 1, but exceeded a value of $KC = 0{\cdot}36$ in Expt. 4, probably because of the larger initial regression error.

Effectiveness of correction procedures within the acceptable counting range

The over-all effectiveness of corrections applied to plaque counts falling within the acceptable counting range is shown by Table 3, within which the essential elements of the experimental analysis are summarized. The slopes of regression lines and the mean ratios of corrected counts conform closely to the ideal values of 0 and 1 respectively.

Table 3. *Essential features of acceptable counting range*

	Poliovirus strain			
	Type 3, Saukett		Type 1, LSc-2ab	
Expt. no.	1	4	2	3
No. of observations	363	273	267	204
Limit value of KC	0·24	$\geqslant 0{\cdot}36$	0·18	0·18
Slope of regression line	−0·032	+0·045	+0·011	−0·080
Mean ratio of corrected counts	1·001	1·008	1·002	0·999
Error variance ($\times 10^3$)	1·81	2·13	2·84	2·59

Table 4. *Estimation of the value of the resolution factor R from counting data*

	Virus strain			
	Saukett		LSc-2ab	
Expt. no.	1	4	2	3
Range of values of KC	0–0·24	0–0·24	0–0·18	0–0·18
No. of obscured plaques considered	211	145	106	102
R				
For experiments	0·1074	0·1054	0·0835	0·0770
For virus strain	0·1066		0·0803	
$\sqrt{R}$	0·326		0·283	

The resolution factor, R

The above analysis shows that correction procedures provide an effective means of estimating true plaque counts. The same results also prove that variation in R is of no practical importance within the acceptable counting range.

It is also of interest that the value of R for a particular virus strain does not change substantially from experiment to experiment. Thus routine correction of observed counts may be based on predetermined values of R. This is shown by

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the close agreement between two estimates of R obtained with each of the two virus strains in different experiments (Table 4). That the value of R must be determined for each virus strain, as might be expected, is shown by the significant differences between the strains considered here ($0{\cdot}02 < P < 0{\cdot}05$).

Counting uncertainty

Where corrections are applied to successive counts of the plaques on individual cultures, fluctuation of ratios of corrected counts about the value of 1 will be due to two main factors: (1) the random placement of plaques on the culture surface, which determines the potential number of plaques which may be obscured by overlapping, and (2) counting uncertainty on the part of the observer.

The first of these, together with the two minor sources of variation (the representation of the true count as a continuous instead of a discrete variate, and the residual bias attributable to the imperfect mathematical representation of plaque overlapping) was common to all the experiments described here. In addition the plaques were counted by the same observer under identical conditions. Systematic differences between viruses in the error variances for data within the acceptable counting ranges may therefore be attributed to differences in counting uncertainty.

The differences found (Table 3) were significant, ($P < 0{\cdot}05$), and indicate that the counting of plaques produced by the LSc-2ab strain of virus was associated with greater uncertainty than the counting of plaques produced by the Saukett strain.

Overlap biases of observed counts which exceed the limit to the acceptable counting range

It is well known that observed plaque counts must eventually become heavily biased by overlapping as plaque number is increased. It is therefore general practice to exclude cultures containing numbers of plaques judged to be too many, even though in fact it might be possible to count them.

Such attempts to limit the overlap bias are inefficient where the bias can neither be predicted nor accurately determined. Clearly, what is regarded as an acceptable number of plaques per culture will vary from one worker to another, and for each worker, from assay to assay and from virus to virus. Since the limit values of KC observed here are probably often exceeded, the relationship between plaque count and overlap bias beyond these limits is of practical importance. This relationship was therefore examined by comparing heavily biased counts observed at the second or third counting times with the estimated true counts for the same cultures, which were calculated by correcting the much smaller biases of the counts observed at the first counting time.

The results (Fig. 2) show (1) that the acceptable counting range is the lower third to half of the possible counting range (which terminates as cultures approach semiconfluency); (2) that the overlap bias increases more rapidly than predicted by the empirically modified model once the limit to the acceptable counting range is exceeded; and (3) that attempts to increase precision by increasing the number of plaques counted on each culture may be defeated by the resulting increase in the overlap bias, which may become as large as 40 %.

The effects of plaque morphology on counting

Morphological differences between the plaques produced by the two virus strains were slight but noticeable, the plaques produced by the LSc-2ab strain being less regular and less sharply defined. These differences might be expected to influence counting but, before attempting to determine the nature of this influence, the factors which the observer considers in attempting to resolve overlapping plaques must be identified.

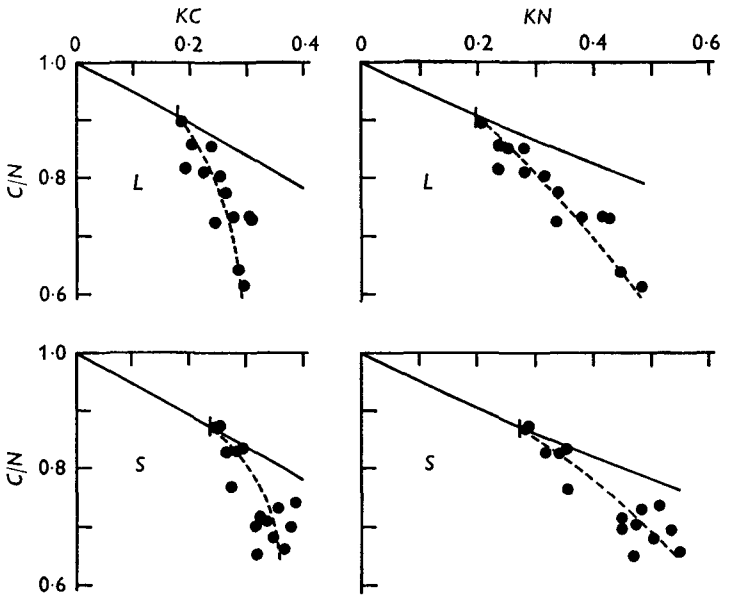

Fig. 2. Overlap biases beyond the limit to the acceptable counting range. True counts (N) for individual cultures were estimated by correcting the counts observed at the first counting time, and the overlap biases of counts (C) observed after plaques had increased in size are shown by the ratio C/N. Data falling within successive 0·01 increments in the range of values of KC were grouped, the mean value of KC was determined, and the corresponding value of KN calculated. Mean ratios were plotted against these values of KN, and the curves (interrupted lines) relating observed values of C/N and KN for the two virus strains LSc-2ab (L) and Saukett (S), were then converted into curves relating C/N and KC. These steps were applied only where values of KC for the later counts exceeded the counting limits, which are shown by the vertical bars.

Calculated overlap biases are shown by uninterrupted lines, and are exceeded once the limit to the acceptable counting range is passed.

Obviously the primary approach is through the analysis of contours and where plaques approach the ideal of sharply defined perfect circles of equal size this is all that is necessary. However, where plaques exhibit irregularities in contours, poor definition of margins and variation in size, resolution by means of contour analysis alone is insufficient. The observer must then attempt to establish a limit to the degree of irregularity which he will accept as representing something more than morphological variation, and must do this for various combinations of plaque size, bearing in mind that different combinations have different probabilities of

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occurrence. For example, where it is difficult to decide whether an area of lysis represents one large irregular plaque, or four small ones of about the same size, the more probable interpretation will usually be accepted, provided the observer is familiar with the concept of probability. Here it is pertinent to note that, where this familiarity does not exist, it has been found that the less probable interpretation is often accepted, and marked 'overcounting', which is accompanied by increased errors, occurs.

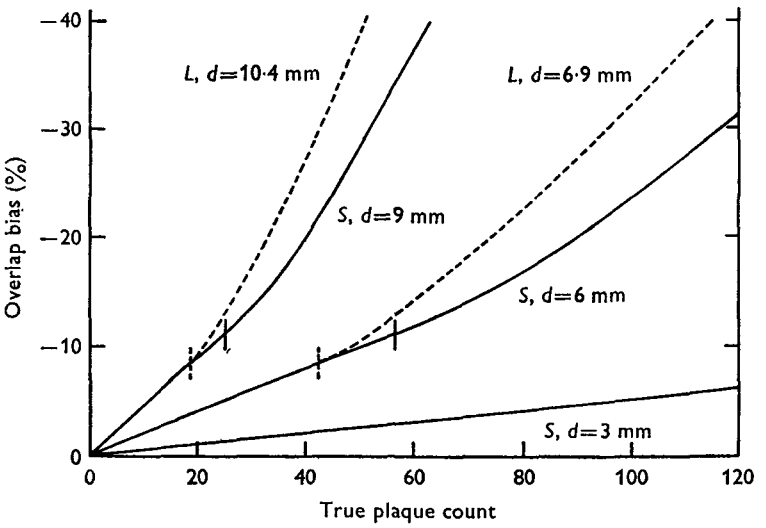

Fig. 3. The relationship between true plaque count and overlap bias for two strains of poliovirus. The single curve relating KN and the overlap bias (C/N) for each virus strain shown in Fig. 2 generates an infinite family of curves when the scale of values of KN is replaced by a scale of values of true counts N, there being one family of curves for each culture area. Three such curves for circular cultures of 60 mm. diameter, and for plaques of mean diameter (d) 3, 6 and 9 mm are shown for the Saukett strain by the solid lines (S), the limits to the acceptable counting ranges being shown by the solid vertical bars. Similar curves (interrupted lines, L) for the LSc-2ab strain, for mean plaque diameters chosen such that the bias of a given true count falling within the acceptable counting range is the same for the two strains, show that counting of LSc-2ab plaques deteriorates earlier (limits to acceptable counting range identified by vertical interrupted lines), so that once this limit is exceeded the overlap bias for a given true count is substantially greater for the LSc-2ab strain than for the Saukett strain.

In the present study the greater uncertainty of counting LSc-2ab plaques is attributed to the less satisfactory morphology of these plaques, and it is very probable that failure of correction procedures at a lower value of KC for this strain, which is shown more clearly in Fig. 3, is attributable to the same cause.

Even though theoretical considerations suggest that uncertain morphology should be accompanied by a deterioration in the ability of the observer to resolve overlapping plaques, the reverse effect was observed, within the acceptable counting range. The value of R was lower for LSc-2ab than for Saukett, which suggests that here increased uncertainty led to a bias towards overcounting.

Of these two effects, counting uncertainty is of greater practical importance, for

the experimentally determined value of R makes allowance for observer bias. Counting uncertainty can be countered either through improvement of plaque morphology or by eliminating consistently poor observers.

DISCUSSION

Simple models such as that advanced here, and the related models advanced by Dulbecco & Vogt (1954) and Cooper (1961), are inadequate since overlapping is treated in terms of the overlapping plaque pair, for which only the distance between centres is critical and orientation need not be considered. This simplification is acceptable for low plaque densities, but more complex forms of overlapping, in which plaques are obscured by the combined effects of two or more neighbours, assume increasing importance as the frequency of overlaps increases. Here the critical distance between centres is greater than that for plaque pairs, and varies both with the number and relative sizes of plaques and with their orientation relative to each other. It is inevitable therefore that such simple models will substantially underestimate the overlap biases of plaque counting.

A more complex model making allowance for this variation in the critical distance might be developed. However, such a model could be valid only if the vagaries of resolution were expressed in precise mathematical terms. At present this does not appear to be possible.

The remaining alternatives are either a wholly empirical approach in which the relationship between observed and true counts is fitted by equations of more or less appropriate form; or an empirical modification of a simple mathematical model to compensate for its theoretical and practical imperfections.

The first approach was adopted by Larsen & Reinicke (1965), who showed that it is feasible to select constants by trial and error. However, the method is too unwieldy to be of use in the routine correction of the overlap biases of observed counts.

The second approach was the one adopted in the present study. The deficiencies of the simple model were overcome by estimating the resolution factor, R, from observed changes in overlap bias which occur on increasing plaque size, instead of using the direct and theoretically correct method of measuring overlapping pairs of plaques at the limit of the resolving power of the observer. In effect, an empirical factor is thus defined which makes allowance for the neglect of the more complex forms of overlapping. Although this allowance cannot be exact, the expected residual biases due to overcorrection in the lower portion of the acceptable counting range, and undercorrection in the upper portion, were shown to be of no practical importance for a substantial part of the possible counting range.

This approach has several advantages over the first: (1) the limit to the number of plaques which may be counted can be determined objectively, and may be simply adjusted for each assay to make allowance for the variations which occur in the mean plaque diameter at the time of counting; (2) corrections for the biases of observed counts are simple to apply; (3) allowance can be made for the partial concealment of the errors of counts (Howes & Fazekas de St Groth, 1969); and

(4) it becomes possible to investigate the effects of plaque morphology and subjective factors on overlap bias and on other errors of plaque counting.

The practice of correction procedures has two stages. First, counting data must be analysed to estimate the value of the resolution factor and the limit to the acceptable counting range. These estimates are then adopted for all standard assays of a particular virus. The actual corrections are simple and routine computations which require only a table of correction factors or a nomogram, and a slide rule or desk calculator. (Howes & Fazekas de St Groth, in preparation).

Whether correction of the overlap biases should be used will depend on the magnitude of these biases and the level of precision required. They are unnecessary where plaques are very small relative to culture area, regular, and clearly defined; they are likely to be necessary where plaques are relatively large, irregular, or poorly defined. Even where plaques are of the latter type, correction procedures might not be needed if plaques are counted early, when still relatively small. However, small size allied with deficiencies in plaque morphology is likely to lead to greater uncertainty in counting and to a consequent loss of precision, while early counting can also lead to a substantial increase in the apparent heterogeneity of cultures with respect to their sensitivity to virus, with a further loss of precision (cf. Howes & Fazekas de St Groth, 1969).

Alternative solutions to the overlap problem have been used or proposed. The first, the 'additive' counting technique, is difficult to justify despite its frequent use. It is inefficient because the small plaques which appear after the first counting time are particularly readily obscured by the large plaques already present, and the resultant overlap biases are variable and difficult to predict. The second approach which could be adopted is to limit the overlap bias by severely restricting the number of plaques on any one culture. The limits which must be imposed in order to reduce the probability that a culture will contain an undetected plaque to less than 0·1 have been calculated by Lorenz & Zoeth (1966), but because no quantitative allowance could be made for the capacity of the observer to resolve overlapping plaques, these are very approximate values. From an economic point of view such low limits would make a return to quantal assay techniques preferable. This is shown in Table 5 where the limits necessary to avoid overlapping are compared with those required where the present correction procedures are used.

A more liberal approach to the choice of counting limits had been suggested by Cooper (1967), who proposed, as a guide, that 100 plaques per plate will give no significant loss by overlapping if the internal diameter of the plate is at least 25 times the average plaque diameter. The overlap bias at this limit, for plaques of 'good' morphology, e.g. the poliovirus plaques studied here, is 3·3 %, which varies in 'significance' with the number of replicate cultures used. Where this is 3, for example, the coefficient of variation for plaque-forming particles is 5·8 % and the overlap bias must be regarded as making a very substantial contribution to the total error.

Cooper's limit can also be used to estimate the practical value of correction procedures. For $R = 0{\cdot}1$ the value of KC corresponding to this limit is 0·065, but

where corrections are applied this can be raised to between 0·18 and 0·24, an approximately threefold increase in the acceptable counting range. Provided all counts within the counting range are equally represented, which is the expectation where data from many assays are considered, 11–12 % of the statistical information will be contained within the lower third of the counting range; 88–89 % in the upper two-thirds which is rejected when corrections are not applied. Correction procedures can therefore give as much as a ninefold increase in the statistical information provided by an assay, and a corresponding threefold increase in precision as measured by the coefficient of variation. The potential practical value of correction procedures is therefore very substantial.

Table 5. *Limits to observed counts for various combinations of plaque and culture diameters*

(Two limits to observed counts are given in each column: a = limit where corrections for overlap biases are applied; for limit $KC = 0{\cdot}2$ and $R = 0{\cdot}1$ (the overlap bias at $KC = 0{\cdot}2$ is 10·4 %). b = limit necessary to reduce probability that a culture will contain an obscured plaque to 0·1 (Lorenz & Zoeth, 1966).)

D† (mm.)		Mean plaque diameter, d (mm.) 1	2	3	4	5	6
30	*a*	450	112	50	28	18	12
	b	10	5	2	.	.	.
40	*a*	800	200	89	50	32	22
	b	13	7	4	2	.	.
50	*a*	1250	312	139	78	50	35
	b	17	9	6	4	2	.
60	*a*	1800	450	200	113	72	50
	b	22	10	7	5	4	.
100	*a*	5000	1250	556	312	200	139
	b	33	17	11	9	7	6

† Culture diameter.

Here it should be noted that, in the few previous studies in which counting data have been examined carefully for the presence of the overlap bias (Cooper, 1961, Berg, Harris & Chang, 1963, and Higgins, 1965, for polioviruses; and Larsen & Reinicke, 1965, for vaccinia virus), it has invariably been found. In the present study it has been effectively measured for the first time.

SUMMARY

The number of plaque-forming units is underestimated if plaques overlap. A simple model was developed to account for this bias, and tested by an extensive experimental analysis. It is shown that models of this type are inadequate, but can be modified to give objective methods for setting limits to the acceptable counting range, and for correcting the overlap bias of observed counts which fall within this range.

Where overlapping of plaques is a significant source of error, these methods will improve the efficiency of plaque assays and will render the statistical analysis of counting data more reliable.

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APPENDIX

Estimation of P by means of repeated counting of plaques

If we consider an ideal system for which a counting time may be chosen such that after plaques have once been counted, no new plaques appear, a decrease in the observed plaque count after an interval of time must be attributed to an increase in the number of undetected plaques, because the true count, N, is constant. This increased frequency of undetected plaques may be due either to an increase in plaque size, or to a change in the morphology of plaques which would result in a change in the value of the resolution factor P, or to both. However, in practice the plaque morphology of polioviruses does not change appreciably with time and any change in the frequency of undetected plaques is assumed to be due to a change in plaque size. From equation (6):

$$N = -\frac{1}{K}\log_e(1-KC) = \frac{1}{K}(KC+\tfrac{1}{2}K^2C^2+\tfrac{1}{3}K^3C^3+\ldots) \tag{10}$$

Since $KC < 0{\cdot}3$, in practice, $N = C+\frac{1}{2}KC^2$ with a maximal error of less than $2{\cdot}5\,\%$ usually less than $1\,\%$.

As N is the same at each time of counting

$$C_1+\tfrac{1}{2}K_1C_1^2 = C_2+\tfrac{1}{2}K_2C_2^2, \tag{11}$$

where the subscripts denote the values at the first and second time of counting. Substituting for K in terms of equation (5), we have

$$P^2 = \frac{D^2(C_1-C_2)}{2(C_2^2d_2^2-C_1^2d_1^2)} \tag{12}$$

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