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STUDIES ON SCHISTOSOMIASIS. 5.* SAMPLING METHODS FOR ESTIMATING THE NUMBERS OF CERCARIAE IN SUSPENSION WITH SPECIAL REFERENCE TO THE INFESTATION OF EXPERIMENTAL ANIMALS

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CONTENTS

ABSTRACT

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Various sampling methods for *Schistosoma mattheei* cercariae, including sampling from different concentrations, were tested. The most satisfactory method consisted of mixing the cercarial suspension by pouring it to and fro between two cylinders and then sampling it by oral suction on a bulb pipette with a large aperture. The aliquots withdrawn were stored in bottles containing formol-saline for later microscopic examination.

This method of sampling was tested extensively. The results showed that the variation between aliquots was larger than that expected for either a binomial or a Poisson distribution, indicating that the cercariae were not randomly distributed in the suspension. Moreover, significant variations occurred between the totals of series of aliquots, possibly indicating uncontrolled factors, e.g. in mixing.

From these results, methods of making up doses of carcariae for infesting sheep and cattle were developed:

The *Sheep Method* is used for doses of up to 15 000 cercariae and is therefore applicable to infestation of primates as well as sheep. Several series of aliquots are made up, some of which (one or more series per animal) are used for infesting the animals concerned (Infestation Series), while others are retained for estimating the infestation doses (Estimation and Additional Estimation Series). One Estimation and one Additional Estimation Series suffice for estimating the numbers of cercariae in up to eight Infestation Series drawn from the same cercarial suspension, which is mixed immediately before each series of aliquots is withdrawn.

In the *Bovine Method*, which is used for doses of over 15 000 cercariae, an Estimation Series and an Additional Estimation Series of aliquots are withdrawn from every dose of cercariae to be used for infesting an animal. Thus the number of cercariae is estimated separately for each animal.

Formulae were compiled for these methods to determine the upper levels (with a specified probability) of the percentage errors in the estimated doses. These formulae were applied to the results and some expected percentage errors in the numbers of cercariae estimated by the two methods, calculated.

A result for the probability distribution of aliquot counts under the assumption of randomness is proved for the first time.

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* See Appendix C for particulars of the other papers in this series

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Introduction

In comparative trials with schistosomes it is necessary to determine as accurately as possible the number of cercariae used for the infestation of individual animals. If small doses of cercariae are required, the total number of parasites can be determined, for instance by counting those present in drops of suspension (Olivier & Stirewalt, 1952). This method is, however, time-consuming and is impractical when large numbers of animals are involved.

If larger numbers of cercariae are required (e.g. for rhesus monkeys, chimpanzees, cattle or sheep) it is impossible to count them all because they swim about and, moreover, their infective life is short (Olivier, 1966). In such cases as well as when many small doses are required, aliquots must be used to determine the number of cercariae in a suspension.

Various methods of mixing suspensions, sampling and counting of aliquots have been used. Usually a constant volume or percentage of the total volume of suspension is sampled (Faust & Hoffman, 1934; Giovannola, 1936; Krakower, Hoffman & Axtmayer, 1940; Abdel Azim & Cowper, 1950; Barbosa, Coelho & Dobbin, 1954; Luttermoser, 1955; Ritchie, Garson & Knight, 1963; Heitmann, 1969), by semi-automatic pipette (Ritchie *et al.*, 1963) or with a syringe (Schubert, 1948). The suspension may be mixed by manual stirring (Krakower *et al.*, 1940), by mechanical stirring (Ritchie *et al.*, 1963), or by pouring back and forth between vessels (Heitmann, 1969).

Counting is usually done in watch glasses (Krakower *et al.*, 1940; Abdel Azim & Cowper, 1950), Petri dishes (Heitmann, 1969) or after filtration through filter paper disks (McClelland, 1965; Pitchford, Meyling, Meyling & Du Toit, 1969).

The accuracy of the different methods of sampling has not been investigated in detail. Faust & Hoffman (1934) and Krakower *et al.* (1940) estimated the accuracy and Schubert (1948) calculated a standard deviation of 15% (of the mean number of cercariae per ml). The numbers of cercariae per aliquot and the number of aliquots examined were not stated.

Only limited numbers of relatively valuable animals like cattle, sheep, monkeys and chimpanzees can be used in experiments. If the number of cercariae used for the infestation of each individual cannot be determined accurately, the variations in worm burdens are so large that it is often impossible to compare different groups of animals and obtain statistically significant results.

The experiments described in this paper were designed to develop a relatively accurate method of estimating the number of cercariae required for the infestation of sheep and cattle. The accuracy of different sampling methods was also evaluated.

General Aspects Concerning Materials and Methods Used

Cercariae

The cercariae used in these investigations were from a strain of *Schistosoma mattheei* obtained from Zululand (McCully & Kruger, 1969) and maintained in the laboratory in sheep and in *Bulinus* (*Physopsis*) *globosus* and *B.* (*P.*) *africanus* as described by Kruger & Heitmann, 1967 and Heitmann, 1969.

The cercariae were collected as described by Heitmann (1969). As cercariae tend to cluster around snail faeces, the suspensions were cleared of faeces by sedimentation.

Mixing of Cercarial Suspensions

The suspensions were mixed by pouring them three or four times from one glass measuring cylinder to another (Heitmann, 1969). Aliquots were taken immediately after mixing. When the aliquots were 10 ml or less, the suspension was mixed before each series was removed; when 50 ml were removed, it was mixed before the first and the fourth aliquots and when 100 ml were withdrawn, before each aliquot.

Sampling

In each experiment a single pipette was used for withdrawing all the aliquots of appropriate volume. In order to prevent cercariae from adhering to the pipette, each aliquot was withdrawn and expelled with the minimum delay.

The aliquots were usually placed in bottles containing 3% formol-saline and stored for up to 60 days before counting the cercariae. When formalin was added to the bottle after the cercariae had been placed in it, they adhered tenaciously to the sides and it was difficult to rinse them out for counting.

Cercarial Counts

These were carried out at 12× magnification* in thinwalled glass Petri dishes** with rounded junctions between the floors and sides. Thin lines 0,5 cm apart were scored with a diamond pencil on the outside of the Petri dishes when low concentrations of cercariae were counted and on the inside when the concentrations were high.

If the dishes have sharp angles, cercariae lying close to the side are distorted and cannot be counted. Dishes with lines scored on the outside are unsuitable for counting high concentrations of cercariae because the lines are slightly out of focus during counting and look wider than they are in reality. Large numbers of cercariae appear to lie in contact with or very close to these lines and are often either counted twice or not counted at all when adjacent lanes are examined for cercariae, especially those near the lines. On the other hand when there are a few cercariae in each dish they are very difficult to see when they are unstained and actually lying on lines scored on the inside of the dish. Particularly with low concentrations of cercariae the dish is moved relatively fast during the counting process and they are easily missed. The ideal would be to stain the cercariae with iodine (Heitmann, 1969) and use Petri dishes scored on the inside only.

Most of the cercariae sank to the bottom of the counting dish. However, as some were found to float on the surface of the fluid and others in contact with the sides of the dish remained in suspension, the surface as well as the depth of the fluid were examined. To prevent optical distortion the dishes were filled to a maximum depth of 0,75 cm (top of water meniscus).

Since cercariae adhered to the glass, the dishes were rinsed two or three times and the entire inner surface of the dish wiped with the finger between counts.

* Wild stereoscopic microscope
** Pyrex, diameter 6,5 cm; depth 1,5 cm

The cercariae to be counted were not stained with iodine for easier counting (Heitmann, 1969) because one of the authors is allergic to iodine. Furthermore, membrane disk filtration techniques (McClelland, 1965; Pitchford *et al.*, 1969) were not used because upon drying they caused distortion of the cercariae and tailless specimens could not then be differentiated from other organisms usually found in the supension (Van Wyk, unpublished data).

When the aliquots were counted, the bottles in which they had been stored were rinsed repeatedly with a strong jet of water to remove all the adherent cercariae. Rinsing continued until no more cercariae were recovered from these bottles.

Experiment 1

Numbers of Cercariae Counted in Aliquots Placed Directly into Petri Dishes Compared to Those Stored in Bottles Before Counting

This experiment was designed to determine the number of cercariae lost when aliquots were placed in bottles for subsequent counting instead of placing them directly into Petri dishes for immediate counting.

Method

Two series of six by 10 ml aliquots were withdrawn from 2 000 ml cercarial suspension. The cercariae in one series were placed in six Petri dishes, killed with formalin and counted immediately. The other six aliquots were placed in bottles containing 3% formol-saline and later washed into Petri dishes for counting.

Results (Table 1)

Table 1 Numbers of cercariae counted in aliquots placed directly into Petri dishes compared with those stored in bottles before counting (Experiment 1)

Series	1	2
Aliquots	Direct	Indirect (Bottles)
1	389	368
2	429	435
3	408	410
4	414	399
5	396	364
6	401	390
Means	406,2	394,3
*S.D.	14,2	26,7
**C.V.	2,92%	5,6%

* S.D. = Standard Deviation
** C.V. = Coefficient of Variation

The total number of cercariae counted in the six samples collected in Petri dishes was 2 437, while 2 366 cercariae were rinsed out of the bottles used for storing the other six samples.

There was no significant difference (according to the t-test) between the mean counts (viz. 406,2 and 394,3 cercariae) obtained by these two methods.

Comment

It appears that there is no significant loss of cercariae when the aliquots are first placed in bottles and that rinsing the bottles therefore effectively recovers all the cercariae.

Statistical Introduction

The distribution of cercariae in a suspension may be random, "better" than random or "worse" than random. The distribution will be better than random if cercariae tend to repel each other and worse than random if there is a tendency for them to cluster or clump together. If the distribution in the liquid is random, the sampling is unbiased, there are no errors in the counting procedures, and the aliquots are a reasonably small fraction of the initial total volume of cercarial suspension (smaller than, say, 0,1), the numbers of cercariae observed in the aliquots will follow a Poisson distribution. In this the parameter is equal to the true mean number of cercariae per aliquot volume as determined by counting all the cercariae in the suspension—assuming no, or few counting errors (Steel & Torrie, 1960). If this condition is not met, the number of cercariae per aliquot will follow a binomial distribution, with the parameters of p = fraction sampled and n = total number of cercariae in the initial suspension*. With the binomial approach it is difficult to verify whether randomness holds as each successive sample represents a different fraction of the volume from which it is taken. The sampling schemes used in this study do not verify randomness.

The variance of a binomial distribution, $np(1-p)$, is smaller than that of the Poisson, np. Therefore it was considered that if the variances obtained were smaller than those expected with a Poisson distribution, they could indicate a binomial distribution. The variances obtained, however, were sometimes larger than expected for a Poisson distribution (see p. 162 and 165) and hence it was not thought necessary to design a sampling scheme for testing for a binomial distribution.

Four experiments were planned to estimate the variations involved and to compare different methods of sampling.

Experiment 2

Sampling with Pipettes of Different Volume

In this experiment a single cercarial suspension was sampled with pipettes of different volume.

Method

Glass bulb pipettes with small apertures of approximately 1 mm in diameter (SA pipettes) were used to withdraw three series of aliquots from 2 000 ml of suspension. One series consisted of ten aliquots of 10 ml and the others of five each of 50 ml and 100 ml.

After withdrawal of the aliquots, the pipettes were rinsed thoroughly and the expelled fluid examined for cercariae.

* See Addendum A for proof

Results (Table 2)

TABLE 2 Sampling with pipettes of different volume (Experiment 2)

Series	1	2	3
Aliquot	Cercarial counts		
	10 ml	50 ml	100 ml
1.....	322	1 430	3 035
2.....	330	1 462	3 154
3.....	336	1 496	2 903
4.....	321	1 561	2 937
5.....	326	1 540	3 067
6.....	312		
7.....	314		
8.....	319		
9.....	316		
10.....	342		
S.D.....	9,74	54,0	101,2
C.V.....	3,01%	3,6%	3,35%
Mean.....	323,8	1 497,8	3 019,2
*C.....	296,0	1 480,1	2 960,0

* C = concentration of cercariae in each pool after sampling

From the cercarial suspension used in this experiment 59 205 cercariae were counted, of which 25 823 were in the aliquots and 33 366 in the residual 1 150 ml of fluid.

When the pipettes were rinsed 3, 2 and 11 cercariae were obtained from the 10, 50 and 100 ml pipettes respectively.

Comments

The number of cercariae adhering to the pipettes was very small in comparison with the number in the aliquots and it may be assumed that the carry-over between aliquots is negligible.

EXPERIMENT 3

COMPARISON OF PIPETTES WITH SMALL AND LARGE APERTURES

It seemed probable that pipettes with large apertures (LA pipettes) would be less deleterious to cercariae than SA pipettes. Aliquots withdrawn with the two types of pipettes as well as different sized LA pipettes were therefore compared.

Method

The tip of a bulb pipette was cut off to increase the size of its aperture to 1,5 mm diameter. Two series each consisting of 10 aliquots of 1 ml were then withdrawn in turn with an SA and an LA graduated pipette.

The remaining 1 980 ml of cercarial suspension were mixed thoroughly and 500 ml poured into a 2 l glass measuring cylinder. This portion, A, as well as the remaining portion, B, was made up to 2 000 ml with water.

LA bulb pipettes were used to withdraw 10 aliquots of 10 ml, five of 50 ml and five of 100 ml from both portions A and B. In addition 10 aliquots were withdrawn from Portion B with a 1 ml graduated LA pipette and 10 with a similar SA pipette.

The third, fourth and fifth 100 ml aliquots of Portion B were examined 60 days after collection.

Results (Table 3)

The total count of this suspension was 45 029 cercariae, of which 20 043 were removed in the aliquots; the counts of the remainders of Portion A and B were 6 011 and 18 975 respectively.

Comments

There was no consistent difference between the percentage error of sample counts drawn with SA pipettes and LA pipettes and as the latter were thought to cause less damage to the cercariae, they were used subsequently.

TABLE 3 Comparison of pipettes with small and large apertures (Experiment 3)

Series	1	2	3	4	5	6	7	8	9	10
Aliquot	Portion (1)		Portion 2 (A)			Portion 2 (B)				
	1 ml *LA	1 ml **SA	10 ml LA	50 ml LA	100 ml LA	1 ml LA	1 ml SA	10 ml LA	50 ml LA	100 ml LA
1	21	29	61	334	606	21	13	176	850	1 626
2	31	18	49	307	579	16	14	169	833	1 620
3	28	26	49	325	550	20	16	161	851	1 648
4	43	18	57	335	594	17	22	170	817	1 762
5	31	19	64	296	604	23	14	157	772	1 583
6	18	22	67	—	—	22	19	162	—	—
7	20	26	57	—	—	14	20	170	—	—
8	40	29	60	—	—	15	14	171	—	—
9	32	31	43	—	—	14	22	181	—	—
10	19	26	68	—	—	23	16	177	—	—
S.D.	8,79	4,83	8,27	17,24	23,08	3,69	3,46	7,58	32,54	68,0
C.V.	31,1%	19,8%	14,4%	5,4%	3,94%	20,0%	20,36%	4,48%	3,95%	4,1%
Mean	28,3	24,4	57,5	319,4	586,6	18,5	17,0	169,4	824,6	1 647,8
C	22,5		55,5	277,5	554,8	16,7		166,9	834,7	1 669,3

* LA = Pipette with a large aperture
** SA = Pipette with a small aperture

TABLE 4 Comparison of manual and "mechanical" withdrawal of aliquots and use of siliconized glassware (Experiment 4)

Series	1	2	3	4	5	6	7	8
**Aliquot	(1) Non-siliconized						(2) Siliconized	
	Mechanical* 10 ml	Manual** 10 ml	Manual 50 ml	Mechanical 50 ml	Mechanical 50 ml	Manual 50 ml	Mechanical 10 ml	Mechanical 50 ml
1	31	23	159	158	154	130	33	120
2	28	21	147	121	153	149	34	130
3	21	34	140	143	132	126	25	107
4	40	28	135	141	125	146	33	126
5	31	32	124	135	129	142	30	141
6	22	27	—	—	—	—	20	126
7	38	39	—	—	—	—	31	121
8	23	20	—	—	—	—	33	131
9	24	28	—	—	—	—	24	112
10	27	19	—	—	—	—	31	124
S.D.	6,55	6,53	13,09	10,6	13,83	10,08	4,74	9,7
C.V.	23,0%	24,1%	9,3%	7,6%	9,98%	7,28%	16,1%	7,8%
Mean	28,5	27,1	141,0	139,6	138,6	138,6	29,4	123,8
C	27,6		137,9				24,6	122,9

* Pipettes filled by means of an attached syringe
** Pipettes filled by oral suction

EXPERIMENT 4

COMPARISON OF MANUAL AND MECHANICAL WITHDRAWAL OF ALIQUOTS AND USE OF SILICONIZED GLASSWARE

In an attempt to obtain more uniform aliquots, a comparison was made of those withdrawn by oral suction on a pipette (manual) with others removed with a syringe on a pipette (mechanical). In addition, as Fife, Sleeman & Bruce (1967) reported that cercariae did not adhere to glassware coated with a silicon layer, some of the glassware used was treated with this substance.

Method

A cercarial suspension was divided into portions of 990 ml (Portion 1) and 1 020 ml (Portion 2). Both portions were made up to 2 000 ml with water.

Ten ml and 50 ml syringes were coupled to 10 ml and 50 ml bulb pipettes with rubber latex tubing. An adjustable mechanical device was attached to the 50 ml syringe for rapid filling.

Aliquots were removed from Portion 1 in the following sequence:

(1) Ten 10 ml samples by syringe (coupled to an LA pipette).
(2) Ten 10 ml samples by oral suction (on an LA pipette).
(3) Five 50 ml samples by oral suction.
(4) Five 50 ml samples by syringe.
(5) Five 50 ml samples by syringe.
(6) Five 50 ml samples by oral suction.

From Portion 2, ten 10 ml and ten 50 ml aliquots were withdrawn by syringe; these were placed in bottles previously coated with silicon* and the cercariae then killed by the addition of formol-saline.

* Dow Corning 772, Dow Corning Corporation, Michigan, U.S.A.

Results (Table 4)

The total cercarial count for the original suspension was 10 426. Of these 4 877 were in the various aliquots and 2 166 and 3 383 in the remainders of Portions 1 and 2 respectively.

Comments

The concentration of cercariae was low in this experiment. There was no consistent difference between the counts obtained by sampling by oral suction and mechanical sampling and as oral suction is easier, it was used in subsequent experiments. Siliconization of glassware did not facilitate removal of cercariae and the use of silicon was therefore discontinued.

EXPERIMENT 5

SAMPLING DIFFERENT CONCENTRATIONS OF CERCARIAE WITH A SINGLE PIPETTE

It seemed probable that the variance between aliquot counts would be dependent upon the concentration of cercariae in the suspension. A cercarial suspension was therefore serially diluted and the cercariae in each of two series of 10 aliquots from every dilution counted.

Method

Two series of ten 10 ml aliquots were taken from 1 000 ml of cercarial suspension. Similar aliquots were withdrawn after twofold, fourfold and sixteenfold dilution of the original suspension.

Results (Table 5)

The results are tabulated in Table 5.

TABLE 5 Sampling different concentrations of cercariae with a single pipette (Experiment. 5)

Series	1	2	3	4	5	6	7	8
Aliquot	(1) Undiluted		(2) Diluted 2×		(3) Diluted 4×		(4) Diluted 16×	
	a	b	a	b	a	b	a	b
1	420	470	233	205	118	120	28	27
2	481	455	220	248	111	115	28	27
3	425	452	194	233	103	94	27	26
4	458	417	224	209	115	112	31	33
5	474	455	205	204	122	101	32	18
6	406	418	205	218	121	95	26	23
7	405	414	208	204	106	95	33	41
8	450	453	235	263	122	102	32	21
9	448	447	245	270	115	115	33	28
10	471	450	242	221	108	108	28	25
S.D.	28,18	19,47	17,58	24,95	6,87	9,75	2,67	6,42
C.V.	6,35%	4,39%	7,95%	10,97%	6,02%	9,05%	8,95%	23,87%
Mean	443,8	443,1	221,1	227,5	114,1	105,7	29,8	26,9

A total of 16 120 cercariae was counted in this experiment. The coefficients of variation were 5,32% for the 20 aliquots from the undiluted suspension and 9,48%; 8,36% and 17,65% from the two-, four- and sixteenfold dilutions respectively.

Comments

As was expected, the percentage error increased with increasing dilution of the suspension.

DISCUSSION OF THE RESULTS OF EXPERIMENTS 1 TO 5

The hypothesis that the variance in the aliquots estimates the variance of a Poisson distribution, was tested with χ^2 tests for each of the different series (Steel & Torrie, 1960, p. 397). These results are presented in Table 6.

TABLE 6 Comparison of variances of Experiments 1 to 5 with those of Poisson distributions

Experiment	Series	Variance	Degrees of freedom	χ^2	True concentration per sample volume†
1	1	201,64	5	2,48	—
	2	712,89	5	9,04	—
2	1	94,87	9	2,64*	296,0
	2	2 916,00	4	15,58**	1 480,1
	3	10 241,44	4	27,14**	2 960,0
3	1	77,26	9	24,57**	22,5
	2	23,33	9	8,61	22,5
	3	68,39	9	10,71	55,5
	4	297,22	4	3,72	277,5
	5	532,69	4	3,65	554,8
	6	13,62	9	6,62	16,7
	7	11,97	9	6,34	16,7
	8	57,45	9	3,05	166,9
	9	1 058,85	4	5,13	834,7
	10	4 624,00	4	11,09	1 669,3
4	1	42,90	9	13,55	27,6
	2	42,64	9	14,16	27,6
	3	171,35	4	4,86	137,9
	4	112,36	4	3,22	137,9
	5	191,27	4	5,52	137,9
	6	101,61	4	2,93	137,9
	7	22,47	9	6,88	24,6
	8	94,09	9	6,84	122,9
5	1	794,11	9	16,10	—
	2	379,08	9	7,70	—
	3	309,05	9	12,58	—
	4	622,50	9	24,63**	—
	5	47,20	9	3,72	—
	6	95,06	9	8,09	—
	7	7,13	9	2,15*	—
	8	41,22	9	13,79	—

* Significant at 5% level

** Significant at 1% level

† Not known in Experiments 1 and 5

No definite pattern emerges from the χ^2 values or the observed variances. In four cases the hypothesis may be rejected at the 1% level in favour of the alternative that the observed variance is larger than is expected if the Poisson distribution (and thus randomness) holds true. In two cases it may be rejected at the 5% level in favour of the alternative that the observed variance is smaller. Although the observed variances were larger than the true mean number of cercariae per sample volume in only 12 of the 21 cases (compare columns 3 and 6 of Table 6), it was thought that the χ^2 results are an indication that the variances tend to be too large, especially if one bears in mind that the χ^2 tests are of relatively low power because of the small numbers of degrees of freedom. (This was confirmed later by more extensive sampling in Experiments 6 to 8. Furthermore, the contribution of counting errors to this deviation was later shown to be negligible—Experiment 9). It was therefore considered unnecessary to design a sampling scheme which would permit a verification of the binomial assumption.

TABLE 7 t values

Experiment	Series No. ***	Degrees of freedom	t
1.....	1		†—
	2		—
2.....	1	9	9,03**
	2	4	0,73
	3	4	1,31
3.....	1	9	2,09
	2	9	1,27
	3	9	0,77
	4	4	5,44**
	5	4	2,89
	6	9	1,50
	7	9	0,27
	8	9	1,04
	9	4	—0,70
	10	4	—0,71
4.....	1	9	0,43
	2	9	—0,24
	3	4	0,55
	4	4	0,38
	5	4	0,13
	6	4	0,18
	7	9	3,20*
	8	9	0,29
5.....	1		†—
	2		—
	3		—
	4		—
	5		—
	6		—
	7		—
	8		—

* Significant at 5% level
** Significant at 1% level
*** See Tables 1 to 5
† — t values could not be calculated because the actual total of cercariae was not determined

The hypothesis that the observed mean number of cercariae per sample is an estimate of the "true" mean number per sample, was tested using a t test for each of the different series. These t values are shown in Table 7. The conclusion was that there are three cases for which this hypothesis must be rejected.

All the above results may indicate erratic deviation from randomness. It is, however, impossible to ascertain whether this is due to *clustering* or *clumping* in the suspension or to *biasses* and *inconsistencies* in the sampling and mixing procedures (see also remarks above on the counting errors—Experiment 9).

Three experiments, using larger and better controlled series of aliquots, were planned in an attempt to verify the above conclusions and to isolate the sources of variation. After the preliminary experiments it appeared that the 10 ml pipette would be used most often in this laboratory for determining numbers of cercariae in suspension for infesting sheep and cattle and 10 ml LA bulb pipettes were therefore used in the following experiments.

EXPERIMENT 6

ONE HUNDRED 10 ML ALIQUOTS; APPROXIMATELY 336 CERCARIAE PER ALIQUOT

In this laboratory cercarial suspensions containing approximately 300 cercariae per 10 ml are most commonly used. This experiment was conducted to determine the sampling error and variance of aliquot counts in a suspension containing approximately 336 cercariae per 10 ml.

Method

Ten series of ten 10 ml aliquots were withdrawn in turn from 2 l of cercarial suspension. The time taken to sample Series 3 to 6 and 8 to 10 was recorded. The cercariae in the residual 1 l were counted as well as those remaining in the measuring cylinders used for mixing.

Results (Table 8)

A total of 65 649 cercariae was counted in this experiment: 33 618 in the various aliquots and 32 031 in the residue and the measuring cylinders. The mean count per aliquot was 336,2 with a standard deviation of 21,35 and coefficient of variation of 6,35%.

The mean time taken for sampling the various series was 92,3 seconds with a range of 84 to 114 seconds.

EXPERIMENT 7

TWO HUNDRED 10 ML ALIQUOTS; APPROXIMATELY 338 CERCARIAE PER ALIQUOT

In this experiment the repeatability of the results obtained in the previous experiment was investigated.

Method

The original volume of cercarial suspension was 2 080 ml. When the first 10 series of ten 10 ml aliquots had been taken, the suspension was mixed by pouring it back and forth between two 2 l measuring cylinders. Two 1 l measuring cylinders were used to mix the residual 1 080 ml of suspension while the remaining 100 aliquots were withdrawn.

After completion of sampling, the four measuring cylinders were rinsed and the washings as well as the residual 80 ml suspension were examined for cercariae. Before the cercariae in the washings were killed for counting, they were examined for motility.

The time taken for sampling each series of aliquots was recorded.

Results (Table 9)

A total of 70 819 cercariae was counted: 67 584 were in the aliquots and 3 235 in the residual 80 ml and the washings of the measuring cylinders.

The mean count per aliquot was 337,97 with a range of 272 to 406, a standard deviation of 26,99 and a coefficient of variation of 7,99%.

TABLE 8 One hundred 10 ml aliquots; approximately 336 cercariae per aliquot (Experiment 6)

Series / Aliquot	1	2	3	4	5	6	7	8	9	10	Mean	S.D. 1–10	C.V. 1–10
a.....	338	316	364	349	366	331	343	321	339	321	338,8	17,36	5,12%
b.....	341	328	340	355	359	338	358	302	281	331	333,3	24,98	7,49%
c.....	313	345	327	337	315	333	323	313	331	310	324,7	11,85	3,65%
d.....	316	330	337	349	344	346	367	350	337	322	339,8	14,83	4,36%
e.....	298	339	348	343	354	335	319	321	342	319	331,8	17,11	5,16%
f.....	320	347	342	375	316	305	349	340	337	307	333,8	21,79	6,53%
g.....	332	353	336	312	330	310	344	353	325	384	337,9	21,95	6,50%
h.....	318	295	337	328	325	361	352	383	357	342	339,8	24,98	7,35%
i.....	287	339	347	370	358	336	325	364	343	352	342,1	23,59	6,90%
j.....	276	333	341	354	332	358	396	361	310	337	339,8	32,00	9,42%
Mean.....	313,9	332,5	341,9	347,2	339,9	335,3	347,6	340,8	330,2	332,5	336,18		
S.D.....	21,38	16,92	9,78	18,66	18,75	17,89	23,13	25,87	21,21	22,92		21,35	
C.V.....	6,81%	5,09%	2,86%	5,37%	5,51%	5,34%	6,65%	7,59%	6,42%	6,89%			6,35%

TABLE 9 Two hundred 10 ml aliquots; approximately 338 cercariae per aliquot (Experiment 7)

Series / Aliquot	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Mean	S.D.	C.V.%
a.....	380	348	383	366	340	361	387	389	374	384	356	406	340	346	365	367	338	313	330	363	361,8	23,15	6,40
b.....	327	367	333	351	340	345	329	360	306	324	284	348	326	317	320	296	351	324	306	311	328,3	21,64	6,59
c.....	334	331	326	323	350	305	325	332	348	310	331	335	319	337	287	324	347	315	329	331	326,9	14,99	4,59
d.....	312	367	323	330	313	322	337	355	324	330	394	337	341	318	320	337	310	322	327	356	333,8	20,81	6,23
e.....	321	340	316	372	369	331	316	374	334	325	357	319	301	341	356	328	334	329	320	334	335,9	20,23	6,02
f.....	344	365	325	334	347	316	313	295	377	348	378	328	284	339	367	343	369	345	304	272	334,7	30,25	9,04
g.....	361	352	328	353	326	330	354	347	338	332	334	298	341	374	336	322	333	308	284	304	332,8	22,07	6,63
h.....	334	322	360	331	287	341	375	340	338	400	313	298	328	377	364	388	345	363	332	337	343,7	28,76	8,37
i.....	323	372	322	369	336	289	381	325	387	312	350	319	357	331	383	375	346	298	274	275	336,2	35,59	10,59
j.....	298	368	333	360	350	326	390	364	344	314	392	354	331	332	386	364	392	332	299	278	345,4	32,86	9,51
Mean.....	333,4	353,2	334,9	348,9	335,8	326,6	350,7	348,1	347,0	337,9	348,9	334,2	326,8	341,2	348,4	344,4	346,5	324,9	310,5	316,1	337,92		
S.D.....	23,71	17,51	20,65	18,08	22,76	20,47	30,41	26,80	25,34	30,84	34,79	31,38	21,25	20,31	31,74	28,51	21,96	18,79	20,48	33,38		26,99	
C.V.%.....	7,11	4,95	6,17	5,18	6,78	6,27	8,67	7,70	7,30	9,13	9,97	9,39	6,50	5,95	9,11	8,28	6,34	5,78	6,60	10,56			7,99%

TABLE 10 One hundred 10 ml aliquots; approximately 1 096 cercariae per aliquot (Experiment 8)

Series / Aliquot	1	2	3	4	5	6	7	8	9	10	Mean	S.D.	C.V. %
a.....	1 136	1 114	1 133	1 016	1 127	1 138	1 060	1 079	1 214	1 124	1 114,1	53,16	4,77
b.....	1 079	1 090	1 073	1 044	1 151	1 120	1 028	1 087	1 139	1 032	1 084,3	42,73	3,94
c.....	1 133	1 015	1 143	1 088	1 107	1 130	1 084	1 146	1 104	1 096	1 104,6	38,65	3,50
d.....	1 030	1 066	1 099	1 089	1 123	1 155	1 093	1 021	1 060	1 015	1 075,1	45,51	4,23
e.....	1 058	1 078	1 086	1 113	1 200	1 117	1 134	1 052	1 153	1 057	1 104,8	48,06	4,35
f.....	1 126	1 133	1 090	1 143	1 188	1 149	1 062	1 108	1 075	1 034	1 110,8	46,16	4,16
g.....	1 167	1 119	1 171	1 103	1 082	1 110	1 077	1 046	1 079	1 083	1 103,7	39,94	3,62
h.....	1 059	1 177	1 166	1 117	1 145	1 104	1 053	1 071	1 021	1 006	1 091,9	59,41	5,44
i.....	1 158	1 113	1 141	1 127	1 044	1 075	1 032	1 029	1 052	1 076	1 084,7	47,03	4,34
j.....	1 170	1 135	1 059	1 077	1 110	1 146	1 063	1 025	1 037	1 085	1 090,7	48,15	4,41
Mean.....	1 111,6	1 104,0	1 116,1	1 091,7	1 127,7	1 124,4	1 068,6	1 066,4	1 093,4	1 060,8	1 096,47		
S.D.....	50,83	44,43	39,62	38,50	46,62	24,40	30,84	40,09	59,70	38,30		46,69	
C.V.%.....	4,57	4,02	3,55	3,53	4,13	2,17	2,89	3,76	5,46	3,61			4,26

The cercariae in the residual portion of suspension were active and their swimming motions were only slightly more sluggish than usual despite the fact that they had been poured back and forth between measuring cylinders more than 84 times.

The mean time taken for sampling the different series was 104 seconds with a range of 96 to 116 seconds.

Experiment 8

One Hundred 10 ml Aliquots; Approximately 1 096 Cercariae per Aliquot

In this laboratory the highest number of cercariae per 10 ml of suspension used for infesting animals is about 1 000. It was inadvisable to extrapolate results of experiments with lower concentrations because, for example, clumping of cercariae could occur in the higher concentrations thus causing larger variations in aliquot counts. It was thus desirable to investigate the variation between aliquots containing large numbers of cercariae.

Method

Two 1 l measuring cylinders were used to mix the 1 075 ml of cercarial suspension. Ten series of ten 10 ml aliquots each were then withdrawn as in the previous experiments.

The residual 75 ml and washings of the measuring cylinders were examined for cercariae as in Experiment 7.

Results (Table 10)

In this experiment a total of 118 386 cercariae was counted: 109 647 were in the sampled 1 000 ml and 8 739 cercariae in the residue and the washings of the cylinders.

The mean count per aliquot was 1 096,47 with a range of 1 006 to 1 214, a standard deviation of 46,69 and a coefficient of variation of 4,26%.

Discussion of the Results of Experiments 6 to 8

1. *Statistical evaluation*

The total variation in the three experiments was compared with the parameters of Poisson distributions by using χ^2 tests (Table 11). In all three cases the total variation was too large to be an indication of randomness.

Table 11 χ^2 values (Experiments 6 to 8)

Experiment	Total variation		Within series variation	
	D.F.†	χ^2	D.F.†	χ^2
6.....	99	134,19*	90	108,04+
7.....	199	425,92**	180	344,95**
8.....	99	196,87**	90	147,52**

† D.F. = Degrees of freedom
\+ Significant at the 10% level
* Significant at the 5% level
** Significant at the 1% level

In each case the total variation was broken down into components ascribable to different sources. This was done by means of analysis of variance procedures (Table 12).

The following conclusions may be drawn:

(i) In all three cases there are significant differences between means of different series. This indicates uncontrolled factors, e.g. inconsistent mixing procedures between series of aliquots, which influence the number of cercariae in the aliquots.

(ii) In Experiment 7 there were significant differences between the means of the aliquots in the different positions in the series. With the aid of Tukey's test (Steel & Torrie, 1960, p. 109) it was determined that only the mean of the first position differed significantly from the others and that none of the other differences approached significance. This result may be due to chance variation.

Table 12 Analysis of variance

	Source of variation	Degrees of freedom	Sums of squares	Mean squares	F	F From table	
						P = 0,05	P = 0,01
Experiment 6.....	Series.....	9	8 797,76	977,53	2,34*	1,99	2,64
	Position...	9	2 491,16	276,80	<1	1,99	2,64
	Error.....	81	33 853,84	417,95			
	Total.....	99	45 142,76				
Experiment 7.....	Series.....	19	27 573,80	1 451,20	3,59**	1,62	1,97
	Position...	9	18 684,00	2 076,00	2,51**	1,99	2,64
	Error.....	171	98 761,00	577,50			
	Total.....	199	140 518,80				
Experiment 8.....	Series.....	9	54 118,21	6 013,13	3,32**	1,99	2,64
	Position...	9	15 014,21	1 668,25	<1	1,99	2,64
	Error.....	81	146 734,49	1 811,54			
	Total.....	99	215 866,91				

* Significant at 5% level
** Significant at 1% level

Even if the observed *within*-series variances are compared with parameters of appropriate Poisson distributions (see Table 11 for the χ^2 values), they are still too large to be an indication of randomness within series (compare the discussion of the results of Experiments 1 to 5).

From these results it is clear that it cannot be assumed that cercariae are randomly distributed in the suspension and therefore the Poisson distribution cannot be used to calculate the percentage error in these sampling procedures.

2. *Infestation of Animals*

The foregoing experiments confirmed experimentally the theoretical expectation that the higher the concentration of cercariae is per aliquot, the smaller the percentage variation between aliquots. Moreover, as is well known from statistical theory, the greater the number of aliquots counted (see No. 3, p. 167 below), the lower is the percentage error in determining the number of cercariae in a suspension. These principles were used to develop methods of making up doses of cercariae for infesting animals.

In this laboratory mainly sheep and cattle are used in experiments on schistosomiasis. Because of the disparity in the numbers of cercariae required for infestation of these animals, two different methods were developed for making up the doses and determining the numbers of cercariae in them. The first (henceforth referred to as the "Sheep Method") is for doses of about 1 500 to 15 000 cercariae and the other (called the "Bovine Method") for larger doses.

(i) *Sheep Method*

This method is applicable to the infestation of primates, such as rhesus monkeys (Vogel & Minning, 1953) and chimpanzees (Sadun, von Lichtenberg, Hickman, Bruce, Smith & Schoenbechler, 1966) as well as sheep.

The animals are infested with one or more series of four to 10 aliquots of cercarial suspension and similar aliquots are collected in formol-saline for the subsequent estimation of the number of cercariae used for infestation.

The available cercarial suspension, cleared of snail faeces, is firstly examined macroscopically to estimate the concentration of cercariae. The capacity of the pipette selected is such that each aliquot will contain no fewer than about 300 cercariae. Pipettes of less than 10 ml capacity are not used.

After mixing the suspension, four or five aliquots are withdrawn and the cercariae counted to determine the mean number per aliquot and so estimate the total number of cercariae available. From this the required dose and the appropriate sized pipette to use for sampling are calculated. Allowance is made in the calculations for cercariae which usually fail to penetrate and those which adhere to the measuring cylinder in which the suspension stands during preliminary counts [see Bovine Method, 2 (ii) below]. If necessary, the cercarial suspension is diluted to conform with the capacity of the pipette to be used. For example in Table 8 (Series 1, Aliquots a to e) the calculated concentration of cercariae is 321 per 10 ml. If 3 500 cercariae are required per animal, the 2 l suspension is diluted to 2 140 ml and six 20 ml aliquots used for infestation. A maximum of 10 aliquots is drawn per series as, firstly, this is the limit tested experimentally and secondly, cercariae tend to swim upwards, especially some schistosome species such as *Schistosoma intercalatum* as noted by Wright, Southgate & Knowles (1972). Larger variations in aliquot counts may occur if the sampling process takes a long time. More than one series may, however, be used for infesting a single animal.

A separate 2 l plastic measuring cylinder with an inside diameter of 9 cm and height of 36 cm* is prepared for each animal. The cylinder is rinsed thoroughly with a stream of tap water and about 200 ml water of the same origin and approximate temperature as the cercarial suspension is placed in it.

After mixing, six 20 ml aliquots (the example above) are placed in separate bottles containing 3% formol-saline. This will be called the *Estimation Series* since the counts of these aliquots will be used to estimate the dose used for infestation. Thereafter a second series of six aliquots, called the *Infestation Series*, is placed into a prepared measuring cylinder; this process is repeated until the required number of doses has been prepared. Finally another series, termed the *Additional Estimation Series*, is placed in bottles containing formol-saline. The pipette is shaken free of fluid after each series has been withdrawn to prevent a carry-over of cercariae.

At a later stage the cercariae in the formol-saline are counted. The actual dose received by each animal is calculated from these counts.

When relatively large doses of cercariae (i.e. 3 500 to 15 000) are required and the concentration of cercariae in the suspension is low, more than one series of aliquots may be used to make up the required dose. Moreover, with due consideration of the labour involved in counting numerous aliquots of cercariae and of the accuracy desired in a specific case, the numbers of aliquots required for the Estimation Series may be calculated [see No. 3 (ii) below on p. 168 and Table 15 and 16].

It has been demonstrated that cercariae in suspension are very resistant to mechanical shock (Kloetzel, 1967). However, until the effect of repeated pouring between cylinders on the infectivity of cercariae has been determined, it is not advisable to withdraw more than eight to 10 series of aliquots (of which two series are for later counting) from a single cercarial suspension. In one instance satisfactory results were obtained when nine series of aliquots from a single suspension were used to infest sheep (Van Wyk, unpublished data) but this must be confirmed in further trials.

When more than eight series are required for infestation, the pool of cercariae is divided into several portions. Each portion of cercarial suspension is treated separately and the first and last series of aliquots from each serve as the Estimation and Additional Estimation Series respectively.

(ii) *Bovine Method*

When 15 000 or more cercariae are required for infesting an animal, the Sheep Method is impractical. In the Bovine Method aliquots of cercariae for counting (Estimation Series) are withdrawn from each dose. This entails the counting of relatively large numbers of cercariae in comparison with previously described methods (Giovannola, 1936; Ritchie *et al.*, 1963; Heitmann, 1969).

The concentrations of cercariae used in the Bovine Method are relatively high and are withdrawn with a 10 ml pipette. After counting the cercariae in four or five aliquots as in the Sheep Method, the volume of suspension required to yield approximately the required dose of cercariae is calculated. This volume is corrected to allow for the aliquots to be withdrawn, plus an additional 2% cercariae which usually fail to

* Kartell Milano, TS 125

penetrate and a further 1% which is lost because they adhere to the measuring cylinder in which the suspension stands while the initial counts are done (Van Wyk, unpublished data). This 1% loss probably does not recur when the dose has been made up (see below) because adherent cercariae would release their hold on the cylinder during infestation, when they are attracted by the presence of the definitive host (Bolwig, 1955).

For example, from the data in Table 10 the concentration of cercariae per 10 ml would be 1 094,5 (mean count of Aliquots a to d in Series 1). If the target dose of cercariae required for infestation is 80 000, 730 ml suspension is required for the dose, plus 100 ml for two series each of five 10 ml aliquots (Estimation Series and Additional Estimation Series), plus another 20 ml to allow for those cercariae that do not penetrate or are lost. The total volume of cercarial suspension required is therefore 850 ml.

The suspension is mixed in glass measuring cylinders and the required 850 ml are poured into one of them. This volume of suspension is mixed, poured into the plastic cylinder* (with inside diameter 8,25 cm, height 46,5 cm) used for infesting cattle (by tail immersion) and five 10 ml aliquots placed in formol-saline (Estimation Series). It is important to withdraw the Estimation Series from the cylinder to be used for infestation so as to prevent loss of cercariae adhering to the cylinder during withdrawal of the aliquots (see above). Thereafter the suspension is again mixed and the Additional Estimation Series of five 10 ml aliquots is removed for subsequent counting.

Usually only the Estimation Series of aliquots is counted and the mean count per aliquot in this series is used to estimate the number of cercariae in the suspension used for infestation. The Additional Estimation Series is taken in case the other aliquots are damaged during counting and handling.

The disadvantage of this method is that it is time-consuming if many animals are to be infested, but this is offset by the fact that the number of cercariae per dose is known with a relatively low percentage error [see Section 3 (i) on p. 167 and Table 13, 14]. Moreover, the aliquots can be counted at any time after infestation.

The Bovine Method is unsuitable for making up low doses of cercariae for the following reasons:

(a) More sheep are usually used in experiments than cattle because they are cheaper. The Bovine Method entails the counting of an estimation series of aliquots for every dose of cercariae, which makes dose estimation a very arduous and time-consuming procedure.

(b) A little of the cercarial suspension is lost when it is poured from one cylinder to another to mix it (see above). In the small volume of suspension generally used for infesting sheep this loss is relatively greater than with the large (bovine) doses.

(c) With the small volume of suspension used for sheep it is impractical and dangerous for the worker to remove the Estimation Series from the large, deep cylinder used for infestation (see above).

(d) If the cercarial suspension is diluted sufficiently to overcome problems (b) and (c), the volume of the aliquots required for the Estimation and Additional Estimation Series is so large, that accurate counting of the cercariae takes too long.

* Kartell Milano TS 872

3. *Estimates of the Percentage Error of Cercarial Doses*

Using well-known statistical concepts and results, formulae may be developed for use in the calculation of percentage errors involved in the sampling schemes proposed in Section 2 above. This is done separately for the Bovine and Sheep Methods. Any specific percentage error will, with a probability P, not exceed the calculated value; P is usually 0,95 (or 95%) but may be arbitrarily chosen beforehand.

For the purpose of the developments below (as well as for the t tests used in previous sections) it must be assumed that the probability distributions of sample counts are reasonable approximations to appropriate normal distributions. This assumption was tested with the counts from Experiments 6, 7 and 8 with the aid of the usual χ^2 "goodness of fit" test (Steel & Torrie, 1960, p. 349). The three χ^2 values were 12,0; 6,8 and 9,6 respectively for the three experiments and it may be concluded that there is no indication of substantial deviations from normality.

We discuss the Bovine Method first [see 2 (ii) above].

(i) *Bovine Method*

The theoretical percentage errors for the Bovine Method of making up doses of cercariae can be calculated by means of the following formula:

$$\text{With a probability P percentage error} \leq \frac{\frac{1}{2} L_B}{C'} . 100\%$$

where L_B is the length of an interval which will, with a specified probability (say P), contain the true mean count (C) per aliquot volume of the pool from which the aliquots were drawn

$$\text{and } L_B = t_{(\alpha,f)} \times \sqrt{S_n^2 \left(\frac{V - nw}{V}\right)}$$

and S_n^2 = an estimate of the variance of the mean of n aliquot counts for a particular concentration of cercariae and aliquot size [see Section 3 (iii) on p. 170 for calculating S_n^2];

f = degrees of freedom of S_n^2;

$t_{(\alpha,f)}$ is the $\alpha.100\%$ ($\alpha = 1 - P$) tabulated t value (from Student's t distribution) at f degrees of freedom, for example 1,98 when the degrees of freedom are over 90 (as in Experiments 6 to 8) and 2,26 when S_n^2 is calculated from ten aliquots, or 2,78 for five aliquots;

V = initial volume of the pool;

w = aliquot volume;

n = number of aliquots drawn in the Estimation Series;

C′ = target cercarial count per aliquot volume for infestation in a particular case.

Example

An Estimation Series of five 10 ml aliquots is drawn from 2 000 ml cercarial suspension. The target mean count per aliquot is 336 and S_n^2 for five aliquots [calculated from Experiment 6 in accordance with Section 3 (iii) on p. 170] is 138,14.

$$\text{Then \% error} \leq \frac{\frac{1}{2} L_B}{336} . 100\%$$

$$\text{where } \tfrac{1}{2} L_B = 1,98 \times \sqrt{138,14 \left(\frac{2\,000 - (5 \times 10)}{2\,000}\right)}$$

$$= 22,99$$

$$\therefore \%\text{ error} \leq \frac{22,99}{336} \cdot 100\% = 6,84\%.$$

Tables 13 and 14 were compiled from the results of Experiments 6 and 8 (95% confidence level):

for n = 1 to 10
and (a) for V = 2 000;
mean count = 336,4 cercariae (Table 13)
and (b) for V = 2 000;
mean count = 1 101,3 cercariae (Table 14).

These errors are applicable to similar volumes and concentrations of cercariae and methods of handling as in the experiments mentioned.

TABLE 13 Bovine Method: Percentage errors associated with various numbers of aliquots. The cercarial pool is 2 000 ml, the target concentration of cercariae is 336 and S_n^2 is calculated from Experiment 6

No. of aliquots in Estimation Series	Percentage error
1	12,62
2	9,44
3	8,11
4	7,34
5	6,84
6	6,48
7	6,21
8	5,99
9	5,82
10	5,68

TABLE 14 Bovine Method: Percentage errors associated with various numbers of aliquots. The cercarial pool is 2 000 ml, the target concentration of cercariae is 1 100 per 10 ml and S_n^2 is calculated from Experiment 8

No. of aliquots in Estimation Series	Percentage error
1	8,46
2	6,51
3	5,71
4	5,26
5	4,97
6	4,76
7	4,60
8	4,48
9	4,38
10	4,30

Derivation of the Formulae

The above formulae were obtained as follows:

If an estimate [S_n^2, to be discussed later—Section 3 (iii), p. 170] is available of the variance of the mean of n aliquot counts for a particular situation (i.e. a particular concentration of cercariae and a particular aliquot size), the length (L_B) of an interval which will, with a specified probability (say P), contain the true mean count (C) per aliquot volume of the pool from which the aliquots were drawn, can be calculated. The length of this interval will depend on the number of aliquots drawn for estimating the dose. Since the mean count of these aliquots will be situated in the centre of the interval, the true mean count will, with a probability P, be not further away from the aliquot mean count than half the length of the interval. Hence the percentage error in mean count estimation for the Bovine Method is, with a probability P, not larger than $\frac{\frac{1}{2} L_B}{C} \cdot 100\%$. This will also be the percentage error of the estimation of the number of cercariae left in the pool (the Infestation Dose), since both L_B and C must then be multiplied by the same factor, namely V*/w, where V* is the volume of the pool after withdrawal of the Estimation Series and w is the aliquot volume.

The interval of which L_B is the length, is the confidence interval of C—a well-known statistical concept. Making use of a "Finite Population Correction" (Steel & Torrie, 1960, p. 416) L_B is calculated as follows:

$$L_B = 2t_{(\alpha,f)} \times \sqrt{S_n^2 \left(\frac{V - nw}{V}\right)}$$ where V is the initial volume of the cercarial pool, n is the number of aliquots in the Estimation Series, $t_{(\alpha,f)}$ is the $\alpha \cdot 100\%$ ($\alpha = 1 - P$) tabulated t value (from Student's t distribution) at f degrees of freedom (f the degrees of freedom of S_n^2) and the other symbols as defined above. [S_n^2 depends on the value of n—see Section 3 (iii), p. 170].

The value of C to be used in the calculation of the percentage error should be equal to the concentration of cercariae per aliquot volume in the whole pool which is, of course, not known. This value of C will yield the true percentage errors of the sampling and estimation procedures. If, however, we substitute for C the target count for infestation in a particular case, say C′, we will get the errors as percentages of the target concentrations and these have a very meaningful interpretation. Hence we can use the following formula for calculations of percentage errors:

$$\text{Percentage error} \leq \frac{\frac{1}{2} L_B}{C'} \cdot 100\%$$

(ii) *Sheep Method*

The formula for calculating the theoretical errors for the Sheep Method of making up doses of cercariae [see 2 (i) above], is as follows:

$$\text{Percentage error (with probability P)} \leq \frac{\frac{1}{2} L_s}{C'} \cdot 100\%$$

where L_s is the length of an interval which will, with probability P, contain the difference between the mean count (known) of the Estimation Series and the mean count (unknown) of the Infestation Series.

And the calculation of L_s can vary as follows:

(a) When the Infestation Series is drawn immediately after the Estimation Series:

$$L_s = 2t_{(\alpha,f)} \times \sqrt{S_{n_1}^2 \left(\frac{V - n_1 w}{V}\right) + S_{n_2}^2 \left(\frac{V - n_1 w - n_2 w}{V - n_1 w}\right)}$$

or

(b) When a known volume, V′, has been withdrawn from the cercarial pool between the Estimation and Infestation Series:

$$L'_s = 2t_{(\alpha,f)} \times \sqrt{S_{n_1}^2 \left(\frac{V - n_1 w}{V}\right) + S_{n_2}^2 \left(\frac{V - n_1 w - V' - n_2 w}{V - n_1 w - V'}\right)}$$

The difference in percentage error as calculated by Formulae (a) and (b) above will be small if V′ is small in comparison with V (see Examples a and b on p. 16 below). [Under most conditions Formula (a) will suffice for the calculations when only the Estimation Series of aliquots is counted]

or

(c) When accuracy is improved by the use of the counts in both the Estimation Series and Additional Estimation Series of aliquots:

$$L''_S = 2t_{(\alpha,f)} \times \sqrt{\tfrac{1}{4}\left\{S^2_{n_1}\left(\frac{V-n_1w}{V}\right) + S^2_{n_3}\left(\frac{V-n_1w-V'-n_2w-V''-n_3w}{V-n_1w-V'-n_2w-V''}\right)\right\} + S^2_{n_2}\left(\frac{V-n_1w-V'-n_2w}{V-n_1w-V'}\right)}$$

where

C' = target cercarial count per aliquot volume for infestation in the particular case;
n_1 = number of aliquots in the Estimation Series;
n_2 = number of aliquots in the Infestation Series;
n_3 = number of aliquots in the Additional Estimation Series;
V = initial volume of the cercarial pool;
V' = volume withdrawn between the Estimation Series and the Infestation Series under test;
V'' = volume of suspension withdrawn between the Infestation Series under test and the Additional Estimation Series;
w = volume of a single aliquot;
$t_{(\alpha,f)}$ and f and S^2_n are as for the Bovine Method formula.

As in the case of the Bovine Method, the percentage errors at 95% confidence level were calculated for the Sheep Method [Formula (a) above] from the results of Experiments 6 and 8:

For $n_1 = 1$ to 10; $n_2 = 1$ to 10

and (a) for $V = 2\,000$ ml;
mean count = 336,4 cercariae (Table 15);

and (b) for $V = 2\,000$ ml;
mean count = 1 101,3 cercariae (Table 16).

TABLE 15 Sheep Method: Percentage errors associated with various numbers of aliquots. The volume of the cercarial pool is 2 000 ml, the target concentration of cercariae is 336 per 10 ml and S^2_n is calculated from Experiment 6

No. of aliquots in Estimation Series	No. of aliquots in Infestation Series									
	1	2	3	4	5	6	7	8	9	10
1.....	16,39	15,76	15,00	14,60	14,36	14,19	14,07	13,98	13,90	13,84
2.....	15,76	13,35	12,45	11,96	11,66	11,46	11,30	11,18	11,09	11,01
3.....	15,00	12,45	11,46	10,94	10,60	10,38	10,21	10,08	9,98	9,89
4.....	14,60	11,96	10,94	10,38	10,03	9,79	9,61	9,48	9,36	9,28
5.....	14,36	11,66	10,60	10,03	9,62	9,42	9,23	9,09	8,98	8,88
6.....	14,19	11,46	10,38	9,79	9,42	9,16	8,97	8,83	8,71	8,61
7.....	14,07	11,30	10,21	9,61	9,23	8,97	8,78	8,63	8,51	8,41
8.....	13,98	11,18	10,08	9,48	9,09	8,83	8,63	8,47	8,36	8,26
9.....	13,90	11,09	9,98	9,36	8,98	8,71	8,50	8,36	8,23	8,13
10.....	13,84	11,01	9,89	9,28	8,89	8,61	8,41	8,26	8,12	8,02

Example

An Estimation Series of five 10 ml aliquots is drawn from 2 000 ml cercarial suspension, then eight Infestation Series of six 10 ml aliquots and finally an Additional Estimation Series of four 10 ml aliquots directly after the last Infestation Series. The target mean number of cercariae per aliquot is 336, $S^2_{n_1} = 138,14$; $S^2_{n_2} = 124,68$; $S^2_{n_3} = 158,33$ (calculated from Experiment 6 as outlined in Section 3 iii on p. 170).

Then $\%\ \text{error} \leq \frac{\frac{1}{2}L_s}{336} \cdot 100\%$

(a) If the Infestation Series under test is the first one withdrawn:

$$\tfrac{1}{2}L_S = 1,98 \times \sqrt{138,14\left(\frac{2\,000-(5\times10)}{2\,000}\right) + 124,68\left(\frac{2\,000-(5\times10)-(6\times10)}{2\,000-(5\times10)}\right)}$$

$$= 31,66$$

$$\therefore\ \%\,\text{error} \leq 9,42\%$$

or

(b) If (for instance) the Infestation Series under test is the fourth one withdrawn:

$$\tfrac{1}{2}L'_S = 1,98 \times \sqrt{138,14\left(\frac{2\,000-(5\times10)}{2\,000}\right) + 124,68\left(\frac{2\,000-(5\times10)-180-(6\times10)}{2\,000-(5\times10)-180}\right)}$$

$$= 31,62$$

$$\therefore\ \%\,\text{error} \leq 9,41\%$$

or

(c) If the information yielded by the Additional Estimation Series is utilized:

$$\tfrac{1}{2}L''_S = 1,98 \times \sqrt{\tfrac{1}{4}\left\{138,14\left(\frac{2\,000-(5\times10)}{2\,000}\right) + 158,33\left(\frac{2\,000-(5\times10)-180-(6\times10)-240-(4\times10)}{2\,000-(5\times10)-180-(6\times10)-240}\right)\right\} + 124,68\left(\frac{2\,000-(5\times10)-180-(6\times10)}{2\,000-(5\times10)-180}\right)}$$

$$= 27,48$$

$$\therefore\ \%\,\text{error} \leq 8,18\%.$$

Derivation of the Formulae

The above formulae were derived as follows:

In the Sheep Method we are interested in the difference between the mean count of the Estimation Series and the (unknown) mean count of an Infestation Series. This difference will determine the magnitude of the error made when the mean count of the Estimation Series is used as an estimate of the infestation dose. Let $\bar{x}_E$ be the mean count (known) of the Estimation Series and $\bar{x}_I$ the mean count (unknown) of the Infestation Series. The length, L_S, of an interval which will contain the difference $\bar{x}_E - \bar{x}_I$ with a probability P, may be calculated as follows:

$$L_S = 2t_{(a,f)} \times \sqrt{S^2_{n_1}\left(\frac{V - n_1 w}{V}\right) + S^2_{n_2}\left(\frac{V - n_1 w - n_2 w}{V - n_1 w}\right)}$$

where n_1 is the number of aliquots in the Estimation Series, n_2 is the number of aliquots in the Infestation Series, V is the initial volume of the pool, w is the volume of a single aliquot and the other symbols have the same meaning as for the Bovine Method.

The error percentage may again be calculated as follows:

$$\text{Percentage error} \leq \frac{\frac{1}{2} L_S}{C'} \cdot 100\%$$

where n_3 is the number of aliquots in the Additional Estimation Series, V″ is the volume withdrawn from the pool between the Infestation Series under test and the Additional Estimation Series and the other symbols as before. The term in braces is always smaller than the first term under the square root sign of L′s if $n_1 = n_3$ and hence L″s < L′s, from which the conclusion that L″s will lead to a smaller percentage error than L′s immediately follows.

(iii) *Calculation of* S^2_n

Lastly we must consider the estimate of the variance, S^2_n, to be used in the above calculations. The counts of the Estimation Series in the case of the Bovine Method do not provide a valid estimate since they do not account for the "between series" variation—an important source of variation as was shown in Table 12. Hence unless several (at least two, preferably more) Estimation Series are drawn from each pool, with the usual mixing between series, some "external" estimate of the variance must be used. This estimate may have been calculated from counts of similar aliquots from another pool with the same, or approximately the same concentration of cercariae. Care must, however, be exercised in the use of such an estimate because any difference in the sampling or mixing techniques may invalidate the use of the external estimate in a particular case.

TABLE 16 Sheep Method: Percentage errors associated with various numbers of aliquots. The volume of the cercarial pool is 2 000 m[1] the target concentration of cercariae is 1 100 per 10 ml and S^2_n is calculated from Experiment 8

No. of aliquots in Estimation Series	No. of aliquots in Infestation Series									
	1	2	3	4	5	6	7	8	9	10
1.....	11,96	10,67	10,20	9,96	9,81	9,70	9,63	9,57	9,53	9,49
2.....	10,67	9,20	8,65	8,37	8,19	8,06	7,97	7,90	7,85	7,80
3.....	10,20	8,65	8,07	7,76	7,57	7,43	7,33	7,26	7,19	7,15
4.....	9,96	8,37	7,76	7,44	7,23	7,09	6,99	6,91	6,85	6,79
5.....	9,81	8,19	7,57	7,23	7,02	6,88	6,77	6,69	6,62	6,57
6.....	9,70	8,06	7,43	7,09	6,88	6,73	6,62	6,54	6,47	6,42
7.....	9,63	7,97	7,33	6,99	6,77	6,62	6,51	6,43	6,36	6,30
8.....	9,57	7,90	7,26	6,91	6,69	6,54	6,43	6,34	6,27	6,21
9.....	9,53	7,85	7,19	6,85	6,62	6,47	6,36	6,27	6,20	6,14
10.....	9,49	7,80	7,15	6,79	6,57	6,42	6,30	6,21	6,14	6,08

The above expression for the calculation of Ls is applicable where the Infestation Series is drawn directly after the Estimation Series. Suppose a volume of V′ has been withdrawn from the pool between the Estimation Series and the Infestation Series under test. The formula for the calculation of Ls can then be made more general by modifying it as follows:

$$L'_S = 2t_{(a,f)} \times \sqrt{S^2_{n_1}\left(\frac{V - n_1 w}{V}\right) + S^2_{n_2}\left(\frac{V - n_1 w - V' - n_2 w}{V - n_1 w - V'}\right)}$$

This implies that the percentage error in the dose of the first animal to be infested from a specific pool differs from that of the dose of the second animal, and similarly for the third and subsequent animals. However, it is clear that if V′ is small in comparison with V, these differences in percentage errors will be small [see Examples (a) and (b) on p. 169 above].

In the description of the Sheep Method mention was made of an Additional Estimation Series drawn after a number of series had been drawn for infestation purposes. By utilizing the information yielded by the counts of this series the accuracy of the estimation may be improved, i.e. the percentage error can be reduced, by calculating L″s as follows:

The foregoing experiments in this paper confirmed the theoretical expectation that the higher the concentration of cercariae sampled, the smaller the percentage variation between aliquots. This implies that the percentage error, as calculated from the aliquots of some given concentration of cercariae, can be used as an external estimate (see above) of the upper limit of the percentage error of some *higher* concentration of cercariae. In the latter instance, however, the experimenter can be "more sure" than the specified confidence level (say 95%) that the percentage error made is less than this upper limit. Conversely the S^2_n value of a given concentration of cercariae can be used for the calculation of an upper limit of the percentage error of some *lower* concentration of cercariae.

$$L''_S = 2t_{(a,f)} \times \sqrt{\frac{1}{4}\left\{S^2_{n_1}\left(\frac{V - n_1 w}{V}\right) + S^2_{n_3}\left(\frac{V - n_1 w - V' - n_2 w - V'' - n_3 w}{V - n_1 w - V' - n_2 w - V''}\right)\right\} + S^2_{n_2}\left(\frac{V - n_1 w - V' - n_2 w}{V - n_1 w - V'}\right)}$$

TABLE 17 Calculation of $\hat{\sigma}^2_E$ and $\hat{\sigma}^2_S$

	Source of variation	Degrees of freedom	Sums of squares	Mean squares	
Experiment 6.....					$\hat{\sigma}^2_E = MS_E = 403,83$
	Series.....	9	8 797,76	$MS_S = 977,53$	
	Error.....	90	36 345,00	$MS_E = 403,83$	$\hat{\sigma}^2_S = (MS_S - MS_E)/10$
	Total.....	99	45 142,76		$= 57,37$
Experiment 7					
	Series.....	19	27 573,80	1 451,20	$\hat{\sigma}^2_E = MS_E = 627,47$
	Error.....	180	112 945,00	627,47	$\hat{\sigma}^2_S = (MS_S - MS_E)/10$
	Total.....	199	140 518,80		$= 82,37$
Experiment 8					
	Series.....	9	54 118,21	6 013,13	$\hat{\sigma}^2_E = MS_E = 1\,797,21$
	Error.....	90	161 748,70	1 797,21	$\hat{\sigma}^2_S = (MS_S - MS_E)/10$
	Total.....	99	215 866,91		$= 421,59$

In the case of the Sheep Method a valid estimate of the variance may be calculated if an Additional Estimation Series was drawn. However, an external estimate calculated from more than two series may be preferable if there is no doubt as to the validity of this estimate for this specific case.

The following formula is applicable for the calculation of S^2_n:

$$S^2_n = \hat{\sigma}^2_S + \frac{1}{n}\hat{\sigma}^2_E$$

where n is the number of aliquots in the Estimation, Additional Estimation or Infestation Series and $\hat{\sigma}^2_S$ and $\hat{\sigma}^2_E$ are calculated from a set of counts consisting of I series and J aliquots in each series (for the sake of simplicity we consider only series consisting of the same number of aliquots). The calculation of $\hat{\sigma}^2_S$ and $\hat{\sigma}^2_E$ is performed by the usual analysis of variance procedure illustrated in Table 17; $\hat{\sigma}^2_E$ being equal to the error mean square (MS_E) and $\hat{\sigma}^2_S$ to $(MS_S - MS_E)/J$, where MS_S is the series mean square. The statistical reasoning leading to this formula is given in Appendix B.

The number of degrees of freedom associated with S^2_n is equal to IJ — I.

EXPERIMENT 9

ESTIMATION OF THE COUNTING ERROR

In the previous experiments the apparent sampling errors of aliquots of cercariae included unknown errors due to manual counting of the cercariae. This experiment was planned to estimate the role played by these counting errors in the variations demonstrated above between cercarial aliquot counts from a given cercarial suspension.

Method

A cercarial suspension was placed in two counting dishes and the numbers of cercariae adjusted to approximate roughly the counts obtained in Experiments 6 to 8.

Thereafter the cercariae in each dish were counted 10 consecutive times without removing them from the dish. Between counts the fluid was agitated to cause a redistribution of cercariae in the dish.

Results (Table 18)

TABLE 18 Estimation of counting error (Experiment 9)

Dish No.	Count No.	Cercariae
1	a	983
	b	973
	c	993
	d	978
	e	971
	f	970
	g	961
	h	975
	i	980
	j	990
	Mean	977,4
	C.V.	0,95%
2	a	391
	b	396
	c	396
	d	396
	e	397
	f	396
	g	396
	h	393
	i	385
	j	399
	Mean	394,5
	C.V.	0,93%

The results are tabulated in Table 18. The percentage counting error was 0,95% for a mean count of 977 cercariae (Dish 1, Table 18) and 0,93% for a mean count of 395 cercariae (Dish 2, Table 18).

Comments

It appears, therefore, that the counting error probably played a minor role only in the percentage variation between aliquot counts from a given cercarial suspension (see reasoning above, in the discussion of Experiments 1 to 5, p. 162).

Conclusions

From the experiments described the following emerged:

While there was an inverse ratio between the concentration of cercarial suspension and the coefficient of variation of aliquot counts, no consistent differences were found between sampling with pipettes having large or small apertures, filling pipettes by oral suction or by an attached syringe, counts of cercariae placed directly into Petri dishes for counting and those stored in bottles for later counting and adherence of dead cercariae to siliconised and unsiliconised bottles.

The following methods of handling cercariae were therefore used in the main experiments (6 to 8): The cercarial suspension is mixed by pouring repeatedly from one measuring cylinder to another. Then a bulb pipette with a large aperture having a capacity of 10 ml or more is used for sampling. The capacity of the pipette is such that each aliquot will contain no fewer than 300 cercariae. It is filled by oral suction and the contents expelled with a minimum delay. The aliquots required for counting are stored in bottles containing formol-saline and counted at a later date.

The variations between the cercarial counts of the aliquots obtained in the various experiments were so large that the requirements for neither the binomial nor Poisson distributions were met, thus indicating that the cercariae were not randomly distributed.

When the variations were broken down into components by means of analysis of variance, significant variations between series were found. This indicated that there were possibly uncontrolled factors e.g. inconsistencies in mixing between series. During the statistical analysis a result for the probability distribution of aliquot counts under the assumption of randomness is proved (Appendix A).

Two methods were developed for making up doses of cercariae for infesting sheep and cattle.

Sheep Method

[Section 2(i), p. 166]. This method is used when dose to up to 15 000 cercariae are required. A series of four to 10 aliquots is used for infesting the animal (Infestation Series) while two similar series are retained for estimating the number of cercariae used for infestation (Estimation and Additional Estimation Series). When the concentration of cercariae is so low that a series of 10 aliquots contains fewer than the required dose of cercariae, more than one series of aliquots can be used for infesting a single animal. Moreover, when more than one animal must be infested, a corresponding number of Infestation Series of aliquots are withdrawn consecutively between the Estimation and Additional Estimation Series.

By using formulae developed for this method [Section 3(ii), p. 168] and with a specified probability the range of variation or percentage error of the estimated dose is calculated. For two concentrations of cercariae the expected errors were calculated from the experimental results for varying numbers of aliquots in the Estimation and Infestation Series (Table 15 and 16).

Bovine Method

[Section 2(ii), p. 166]. When more than 15 000 cercariae are required for infesting an animal, the Bovine Method is used; an Estimation and an Additional Estimation Series are withdrawn consecutively from every dose of cercariae used for infesting a single animal. They are withdrawn *after* this dose has been placed in the container used for the infestation. Hence in this method the dose of every animal is determined separately.

As in the case of the Sheep Method a formula was developed [Section 3(i), p. 167] for calculating the percentage error of each dose of cercariae with a specified probability. In Tables 13 and 14 the expected percentage errors (95% probability) in the estimation of the infestation dose are listed for two concentrations of cercariae respectively (from Experiment 6 and 8).

The numbers of cercariae sampled in both methods are large in comparison with those required for infesting rodents, with the result that they will probably not be applicable to infestation of small laboratory animals.

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APPENDIX A

The Distribution of the Number of Cercariae in an Aliquot

We consider the drawing of an aliquot from a specified pool as sampling without replacement from a population of N individuals. An "individual" is defined as being either a cercaria or a quantity of water with the same volume as a cercaria. The population may thus be considered as consisting of two kinds of individuals, T of the first kind (the total number of cercariae in the pool) and N–T of the second kind (the number of quantities of water as defined above). Consider an aliquot of n individuals where n is a specified proportion of N, say $n = fN$; $0 < f < 1$. The probability that this sample will contain t cercariae is, according to the hypergeometric distribution (Kendall & Stuart, 1963, p. 133) given by:

$$P(t) = \binom{T}{t} \binom{N-T}{n-t} \Big/ \binom{N}{n}$$

and hence it follows that

$$P(t) = \binom{T}{t} \frac{(N-T)!\,(N-n)!\,n!}{(N-T-n+t)!\,(N-t)!\,N!}$$

$$= \binom{T}{t} \frac{(N-n)(N-n-1)\ldots(N-n-T+t+1)\,n(n-1)\ldots(n-t+1)}{N(N-1)\ldots(n-T+1)}$$

$$= \binom{T}{t} \frac{\left(1-\frac{n}{N}\right)\left(1-\frac{n}{N}-\frac{1}{N}\right)\ldots\left(1-\frac{n}{N}-\frac{T-t+1}{N}\right)\left(1-\frac{1}{n}\right)\ldots\left(1-\frac{t-1}{n}\right)\left(\frac{n}{N}\right)^{t}}{1\left(1-\frac{1}{N}\right)\ldots\left(1-\frac{T-1}{N}\right)} \quad \ldots\ldots(1)$$

Now let N and n both tend to infinity so that $\frac{n}{N}$ tends to f (T remaining finite). Then (1) becomes

$$\binom{T}{t} f^{t} (1-f)^{T-t} \quad \ldots\ldots(2)$$

which is the $(t+1)'$ th term of a binomial distribution with parameters f and T; f being the fraction of the pool sampled and T the number of cercariae in the pool. (If only N tends to infinity, it is well known that the hypergeometric distribution reduces to a binomial distribution with parameters n and p, where p is the proportion of individuals in the population which are cercariae).

If F is sufficiently small and T large, a Poisson distribution with parameter fT (the true mean number of cercariae per sample) will be a good approximation to the binomial distribution with parameters T and f. Attention was drawn to this fact in the Statistical Introduction on p. 159).

Since a cercaria is very small, N as defined above, will be very large—even for a pool with a relatively small volume. For the same reason n will also be very large—even for a small aliquot. It thus appears that the binomial distribution derived by letting N and n tend to infinity, should be a good approximation for cercarial sampling, provided that the aliquots are random samples from the pool, i.e. in the absence of any external factors such as inconsistent or insufficient mixing procedures.

T is usually quite large in the case of cercarial sampling and a normal distribution with mean fT and variance $f(1-f)\,T$ will provide an excellent approximation to the binomial distribution.

APPENDIX B

Derivation of the Formula for Calculating S_n^2

The count of the j'th aliquot in the i'th series, say Y_{ij}, is considered as the sum of a number of components, namely

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where μ is the true mean count per aliquot volume in the pool from which the aliquot was drawn, α_i is a deviation from the mean count peculiar to the i'th series and e_{ij} is a further deviation (called the error) peculiar to the particular aliquot. We further consider the series deviations and aliquot deviations to be random and independent variables with variances σ_S^2 and σ_E^2 respectively and zero means. This model is suggested by the analyses in Table 12 of Experiments 6, 7 and 8 and by the fact that there is no consistent pattern in the means of the series in these experiments and that this observation indicates randomness of the deviations of the individual series.

We now require estimates of $\hat{\sigma}_S^2$ and $\hat{\sigma}_E^2$ which will be denoted by $\hat{\sigma}_S^2$ and $\hat{\sigma}_E^2$ respectively.

A set of data consisting of I series and J aliquots in each series may be analysed according to the above model (Graybill, 1961, p. 342):

TABLE 1

Source of variation	Degrees of freedom	Mean square	E (Mean square)
Series.....	I — 1	MS_S	$\sigma^2_E + J\,\sigma^2_S$
Error.....	I(J — 1)	MS_E	σ^2_E
Total.....	IJ — 1		

The "mean square" column is an estimate of the last column. We thus have $\hat{\sigma}^2_E = MS_E$ and $\hat{\sigma}^2_S = (MS_S - MS_E)/J$. In Table 17 these estimates are calculated for Experiments 6, 7 and 8.

In the Bovine Method the mean count of a single series of n aliquots may be used as an unbiased estimate of the mean count N in the pool. To prove this the mean count of the i'th series is

$$\overline{Y}_i = \frac{1}{n} \sum_{j=1}^{n} Y_{ij}$$

The mathematical expectation of this mean is

$$E(\overline{Y}_i) = \tfrac{1}{n} \sum_i E(Y_{ij}) = \tfrac{1}{n} \sum_j E(\mu + a_i + e_{ij})$$
$$= \mu, \text{ (since } E(a_i) = E(\overline{e_{ij}}) = 0)$$

i.e. the mean count in the pool. The variance of this estimate is

$$\text{var}\left(\frac{1}{n} \sum_j Y_{ij}\right) = \frac{1}{n^2} \text{var}\left(\sum_j Y_{ij}\right)$$
$$= \frac{1}{n^2}\left[\sum_j (\text{var } Y_{ij}) + \sum_{j' \neq j}\left\{\text{cov}\,(Y_{ij}, Y_{ij'})\right\}\right]$$
$$= \frac{1}{n^2}\left[n\,(\sigma^2_S + \sigma^2_E) + n(n-1)\,\sigma^2_S\right]$$
$$= \sigma^2_S + \tfrac{1}{n}\,\sigma^2_E$$

and this is estimated by

$$S^2_n = \hat{\sigma}^2_S + \tfrac{1}{n}\,\hat{\sigma}^2_E$$

This same formula is used to determine S^2_n for the Sheep Method, including those instances where an Additional Estimation Series is drawn and counted. In this last instance the formula for the calculation of L″ makes allowance for the fact that the mean count is estimated from two series.

APPENDIX C

List of Previous Publications in this Series

VAN WYK, J. A., 1971. Studies on schistosomiasis. 1. An improved method of concentrating miracidia. *Jl S. Afr. vet. med. Ass.*, 42, 169–170.

VAN WYK, J. A., 1971. Studies on schistosomiasis. 2. An improved technique for counting ova and cercariae. *Jl S. Afr. vet. med. Ass.*, 42, 171–173.

DU PLESSIS, J. L. & VAN WYK, J. A., 1972. Studies on schistosomiasis. 3. Detection of antibodies against *Schistosoma mattheei* by the indirect immuno-fluorescent method. *Onderstepoort J. vet. Res.*, 39, 179–180.

VAN WYK, J. A., 1973. Studies on schistosomiasis. 4. Differential staining of live and dead cercariae after immobilisation with physostigmin. *Onderstepoort J. vet. Res.*, 40, 23–30.

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