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A TECHNIQUE FOR THE RECOVERY OF NEMATODES FROM RUMINANTS BY MIGRATION FROM GASTRO-INTESTINAL INGESTA GELLED IN AGAR: LARGE-SCALE APPLICATION

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ABSTRACT

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A gelled-agar technique for worm recovery was adapted to facilitate the recovery of larval and adult nematodes from the total ingesta of large numbers of sheep. The technique was also used to recover nematodes from 4 calves.

In one trial involving 120 sheep, 100% of 2 013 4th stage larvae (L4) and 92,1% of 134 205 adult *Haemonchus contortus* migrated from the agar preparations. Highly significantly more male than female worms failed to migrate.

Using 1×1/10 aliquot to estimate the numbers of worms that failed to migrate from the agar, the mean error in the total worm count (worms that migrated plus those that failed to migrate) per sheep was 2,2%; with an examination of 2×1/10 aliquot the error was 1,7%.

We concluded from this that the gelled-agar method may be of value for quantitative worm recovery, for example, in anthelmintic tests.

In a second trial, 98,5% of 17 056 L4 and adult nematodes of 5 genera migrated from the ingesta of 4 calves and 96,4% of 62 597 L4 and adult nematodes of 9 species from the ingesta of 15 sheep.

In general, L4 migrated slightly more efficiently than adult worms. In sheep and, to a lesser extent, in calves, *Haemonchus* spp. did not migrate as efficiently as the other genera such as *Ostertagia*, *Trichostrongylus*, *Nematodirus*, *Oesophagostomum*, *Marshallagia* and *Chabertia*.

Résumé

UNE TECHNIQUE POUR LE RECOUVREMENT DE NÉMATODES DES RUMINANTS PAR MIGRATION DU CONTENU GASTRO-INTESTINAL GELIFIÉ: APPLICATION À LARGE ÉCHELLE

Une technique d'agar gelifié pour le recouvrement des vers a été adaptée pour faciliter le recouvrement des nématodes larvaires et adultes de l'ingesta total de grands nombres de moutons. La technique a également été utilisée pour recouvrer les nématodes de 4 veaux.

Dans une expérience comprenante 120 moutons, 100% de 2013 larves de quatrième stade (L4) et 92,1% de 134 205 Haemonchus contortus *adultes émigrèrent hors des préparations d'agar. Un nombre significativement plus élevé de vers mâles que de vers femelles n'émigrèrent pas.*

En utilisant un aliquote de 1×1/10 pour estimer les nombres de vers n'ayant pas émigré de l'agar, l'erreur moyenne dans le total du compte (vers ayant émigré plus ceux ne l'ayant pas fait) par mouton fut de 2,2%; avec un examen d'une aliquote 2×1/10, l'erreur fut de 1,7%.

De ceci, nous avons tiré la conclusion que la méthode d'agar gelifié peut avoir une valeur pour le recouvrement quantitatif de vers, par exemple, dans les tests anthelmintiques.

Dans un second essai, 98,5% de 17 056 L4 et nématodes adultes de 5 genres émigrèrent de l'ingesta de 4 veaux et 96,4% de 62 597 L4 et nématodes adultes de 9 espèces de l'ingesta de 15 moutons.

En général les L4 émigrèrent d'une manière légèrement plus efficiente que les vers adultes.

Chez les moutons, et d'une manière moindre chez les veaux, Haemonchus *spp. n'émigra pas d'une façon aussi efficiente que les autres genres tels que* Ostertagia, Trichostrongylus, Nematodirus, Oesophagostomum, Marshallagia *et* Chabertia.

INTRODUCTION

Jørgensen (1975) made use of gelled-agar preparations to recover infective lungworm larvae (L3) from herbage washings. Van Wyk & Gerber (1978) applied this principle to the recovery of nematodes from small aliquots of the gastro-intestinal ingesta of sheep. Van Wyk (1978) subsequently described a method by which the gelled-agar technique could be applied to large-scale recovery of worms in, for example, anthelmintic trials.

This paper describes in detail the technical aspects of 2 trials in which the suggested modifications (Van Wyk, 1978) were tested. Other aspects of each trial, concerning possible effects of cryopreservation on infective larvae of nematodes, are published elsewhere (Van Wyk & Gerber, 1980a; Van Wyk & Gerber 1980b).

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Some of the modifications tested in Trial 1 (the first trial in which recovery of nematodes was attempted from large amounts of ingesta of numerous sheep) proved cumbersome and impractical and necessitated further modifications in Trial 2.

Through analysis of the worm counts of 240 aliquots of ingesta containing worms that failed to migrate from the agar, attention was paid to the theoretical errors expected in the use of small aliquots to estimate the numbers of worms remaining in the ingesta.

EXPERIMENT 1

Experiment 1 was designed:

(1) to investigate large-scale recovery of nematodes from gelled ingesta, including observations on the expected error associated with the use of small aliquots to determine the numbers of worms that failed to migrate from the agar, and

(2) to investigate possible influences of cryopreservation of larvae on the migration of their progeny.

Materials and Methods

Experimental sheep

Dorpers and Merinos of both sexes were divided into 12 groups of 10 sheep each.

Strains of H. contortus

One of the benzimidazole-resistant strains of *H. contortus* (the Boshof strain, Table 1) originated from Boshof in the Orange Free State (Berger, 1975) and was passaged once in the laboratory by Berger (Coopers, Kwanyanga, East London) before being used in these investigations.

The other resistant strain (O.P.-M. strain) was isolated during January 1977 from Kaalplaas, an experimental farm adjacent to Onderstepoort, when goats died from haemonchosis despite having been treated shortly before with mebendazole* (Van Wyk, unpublished data, 1977).

For comparing the migration from agar of the progeny of frozen and unfrozen larvae, the strains were maintained in donor sheep, using larvae which had never been exposed to freezing in the gas phase of liquid nitrogen ("unfrozen substrains") and, in addition, some larvae of each strain were frozen for later comparison of their progeny ("frozen substrains") with the progeny of the unfrozen substrains. Unfortunately, in the trial a benzimidazole-susceptible strain (B.S.S., unfrozen) was used by mistake in the place of the unfrozen O.P.-M. strain (Van Wyk & Gerber, 1980a), with the result that no O.P.-M. unfrozen L3 were used in the trial.

Some of the larvae of the Boshof strain were frozen in the gas phase of liquid nitrogen (Van Wyk, Gerber & Van Aardt, 1977), and, after thawing, were used to infest a sheep. Larvae from this sheep were, in turn, frozen, thawed and used to infest a further sheep until the cycle had been repeated 5 times. Larvae obtained from the last passage were not frozen before being used in the trial.

After collection the L3 used in the trial were stored at 4 °C and were younger than 3 weeks at infestation.

The experimental design of the trial is shown in Table 1.

Worm recovery

The contents of the abomasa (collected at necropsy after the sheep had been fasted, as described by Reinecke, 1973) were rinsed with physiological saline into a 2 ℓ glass jar prominently graduated at 810 mℓ. As there was usually more than 810 mℓ of abomasal ingesta plus saline, the jar was placed in a water-bath at 40 °C until the ingesta had sedimented to below the 810 mℓ mark, and the supernatant decanted into a bottle for later examination. The contents were then adjusted with 0,9% saline to the 810 mℓ mark and 270 mℓ of 2,6% bacteriological agar suspension added to give a final concentration of 0,65% agar. The mixture was allowed to gel in 3 framed moulds of the type described by Van Wyk & Gerber (1978), consisting of wiremesh supported in perspex frames with removable base plates. Before the frames were filled, the joints between the frames and the base plates were sealed with agar to prevent leakage.

After overnight incubation in saline, the frames were removed from the water-bath, and, while being gently wiped by hand, they were rinsed to remove attached worms. The agar was melted and the hot suspension washed with hot water from a shower spray through a 38 μm sieve to separate the ingesta from the agar.

Both the sediment on the sieve and the saline in which the frames had been incubated were retained separately for worm recovery.

The abomasal mucosae were digested as described by Reinecke (1973).

Total worm counts of the mucosal digests and of the supernatant which was poured off before the ingesta were gelled in agar were done microscopically; 9/10 of the material containing worms which had migrated from the agar slabs was examined macroscopically and 1/10 microscopically.

Two × 1/10 aliquots of the ingesta recovered from the agar slabs were examined microscopically and the remaining 8/10 macroscopically.

TABLE 1 Experiment 1: Experimental design

Day	No. of *H. contortus* L3 dosed			
	Boshof frozen (Groups B, D & F)	Boshof unfrozen (Groups A, C & E)	Onderstepoort resistant, frozen (Groups H, J & L)	Benzimidazole susceptible, unfrozen (Groups G, I & K)
−24	1 128	956	1 274	1 192
−23	1 128	956	1 274	1 192
−22	1 128	1 472	1 028	1 192
Total L3	3 384	3 384	3 576	3 576
0	Treated Groups A, B, G and H with thiabendazole, Groups C & D with mebendazole and Groups I & J with fenbendazole; Groups E & F and Groups K & L remained as untreated controls			
+14	Killed Group G			
+15	Killed Groups H & J			
+17	Killed Groups I, K & L			
+21	Killed Groups A, B & C			
+23	Killed Groups D, E & F			

* Multispec (Ethnor)

TABLE 2 Experiment 1: Percentage migration of adult *H. contortus* from agar gel

Sheep No.	Group A		Group B		Group C		Group D		Group E		Group F		Group G		Group H		Group I		Group J		Group K		Group L	
	Total* worms	Migration (%)**	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)	Total worms	Migration (%)
1	1 207	80,4	2 019	90,3	444	88,7	948	91,4	827	94,1	1 867	90,7	0	—†	2 715	93,9	0	—	34	85,3	1 739	92,0	2 484	96,9
2	1 221	92,1	2 220	93,0	169	92,9	563	92,0	1 315	85,0	2 025	95,1	6	100,0	1 900	94,0	0	—	865	85,0	1 702	95,7	1 398	90,1
3	483	92,8	448	90,4	435	86,7	700	94,3	2 118	94,2	2 285	91,1	27	100,0	1 925	90,1	0	—	1 039	92,3	1 252	85,4	2 076	95,1
4	1 093	88,5	2 495	92,5	74	90,5	1 720	96,6	883	93,3	2 295	93,4	6	83,3	2 369	92,2	0	—	1 099	93,3	1 950	96,8	2 174	96,7
5	1 483	93,2	1 108	91,0	251	92,4	653	89,3	1 903	89,1	1 949	89,1	3	66,7	1 694	87,0	2	100,0	820	100,0	2 186	96,0	2 019	98,2
6	1 006	91,0	673	91,8	514	87,2	385	95,8	1 163	90,9	34	76,5	3	100,0	2 053	88,4	0	—	708	90,5	1 478	93,0	2 198	83,4
7	889	88,6	1 859	91,3	228	83,3	1 692	90,1	955	89,2	2 333	90,4	11	100,0	2 222	91,9	1	100,0	2 108	95,7	2 459	94,9	2 036	86,8
8	1 423	94,1	1 735	92,5	701	96,6	535	96,5	730	95,8	1 369	93,8	1	100,0	1 823	83,3	1	100,0	612	95,4	1 404	88,4	2 181	91,8
9	972	91,1	1 992	92,7	824	97,3	974	93,7	1 165	93,5	1 740	94,2	31	100,0	2 740	95,3	0	—	1 202	98,8	1 632	87,3	2 013	91,0
10	441	92,1	1 146	94,8	197	93,4	447	93,5	516	89,9	1 984	92,5	296	91,2	1 763	94,7	0	—	709	91,3	813	96,3	400	96,0
Mean	1 021,8	90,4	1 569,5	92,1	383,7	90,9	861,7	93,3	1 157,5	91,5	1 788,1	90,7	38,4	93,5	2 120,4	91,1	0,4	100,0	919,6	92,8	1 661,5	92,6	1 897,9	92,6
S.D.***	349,7	4,0	685,2	1,4	244,2	4,5	484,3	2,6	508,1	3,3	681,7	5,3	91,2	11,6	379,9	3,9	0,7	0	529,1	5,1	470,3	4,2	592,8	4,9

* The total No. of adult worms in the agar slabs

** The percentage of worms which migrated from the agar

† Not applicable because no worms were recovered from these sheep

*** Standard deviation

In each sample the first 20 worms encountered (or 50 for samples containing larvae) were identified, but if fewer than 20 or 50 respectively were recovered, all were identified. During the course of the identifications it was noticed that more male worms failed to migrate from the agar gel than females and subsequently the percentages of male worms that migrated from the agar and those that remained in the agar were determined for 55 of the sheep.

Statistical evaluation

The Wilcoxon Matched-Pairs Signed-Ranks Test (Siegel, 1956) was used for comparing the ratio of male and female *H. contortus* that failed to migrate from the agar and the Mann-Whitney U-test (Siegel, 1956) was used for comparing total migration of the progeny of frozen and unfrozen L3.

From the worm counts obtained in the 240×1/10 aliquots (2 aliquots of abomasal ingesta residue per sheep) of the abomasal ingesta of all the sheep, the theoretical errors that can be expected when various numbers of worms remain in the agar were calculated by the method outlined in the Appendix.

Results

Worm migration

All of the 2 013 L4 *H. contortus* and 92,1% of the 134 205 adults that were gelled in agar migrated. While 36,9% (±12,0) of the adult *H. contortus* that migrated from the agar gel were males, 70,8% (±13,5) of those that failed to migrate were males. The difference was highly significant ($P<0,00003$).

The mean migration of adult worms per group of sheep varied from 90,4% (±4,0) to 93,5% (±11,6), with 100% migration from Group I, from which only 4 worms were recovered (Table 2). The mean number of adult worms per group varied from 0,4–2 120,4.

Fewer than 90% of the worms migrated from the ingesta in only 24 out of 112 sheep from which worms were recovered (Table 2).

While the mean migration of adults of the Boshof frozen substrain (Groups B, D & F) was 92,01% (±3,56), that of the Boshof unfrozen substrain (Groups A, C & E, Table 2) was 90,93% (±3,82) and that of the O.P.-M. frozen substrain (Groups H, J & L, Table 2) was 91,25% (±5,99). The differences in migration of the 2 frozen substrains compared with the unfrozen Boshof substrain were not significant ($P>0,05$). This applied also when only the untreated control groups (Groups E and F, Table 2) were compared.

The accuracy of small aliquots for estimating the number of worms that failed to migrate from the agar gel

When 1×1/10 aliquot was examined, the mean percentage error in the estimates of worms remaining in the agar was 34,5% [range 4,7% (143 worms remaining)—114,3% (3 worms)]. If Group G, with a mean of less than 30 worms remaining in the agar is excluded, the mean error was 26,5% (Table 3).

With examination of 2×1/10 aliquots, the mean error was 24,6% [range 3,7% (123 worms)—42,9% (3 worms)]; with Group G excluded, the mean error was 22,8%.

The mean error in the total worm count per sheep was 2,17% when 1×1/10 aliquot was used to estimate the numbers of worms that failed to migrate from the gel; with 2×1/10 aliquots the error was 1,72%.

Comment

In the present experiment all the abomasal ingesta were sedimented to the same volume, but this was impractical because of large variations between sheep in the volume of abomasal ingesta. In some instances the graduated volume was too small and the period of sedimentation had to be extended; in others the volume was too large, in which case fewer than 3 frames would have been sufficient for processing the ingesta. As a result the technique was made more flexible in this respect.

Percentages of adult *H. contortus* that migrated from the agar were similar to those recorded by Van Wyk & Gerber (1978), who tested only aliquots from small numbers of sheep rather than total ingesta from large numbers of animals. For an unknown reason L4 migrated from the agar gel more efficiently than before.

TABLE 3 Experiment 1: *H. contortus* remaining in agar—the accuracy of aliquots used to estimate the number of worms that failed to migrate from the agar gel

Group	Mean number of worms remaining in agar*	Percentage error of estimate of worms remaining in agar					
		Mean error		Maximum error (No. of worms concerned)		Minimum error (No. of worms concerned)	
		1/10 aliquot	2×1/10 aliquots	1/10 aliquot	2×1/10 aliquots	1/10 aliquot	2×1/10 aliquots
A	99 (±56)	15,7	20,7	54,6 (97)	50,8 (126)	− 1,0 (101)	0 (35)
B	123 (±56)	8,6	3,7	63,6 (55)	63,6 (55)	− 7,0 (43)	− 3,9 (156)
C	31 (±21)	42,4	24,6	163,2 (19)	110,5 (19)	−31,0 (58)	13,6 (66)
D	59 (±44)	41,2	36,9	275,0 (16)	150,0 (16)	− 2,4 (82)	19,8 (167)
E	101 (±61)	59,2	41,3	125,8 (31)	109,7 (31)	6,8 (103)	11,7 (103)
F	141 (±68)	22,1	19,3	150,0 (8)	150,0 (8)	15,6 (173)	−10,7 (224)
G	3 (± 8)	114,3	42,9	900,0 (1)	400,0 (1)	53,8 (26)	15,4 (26)
H	182 (±63)	26,7	29,7	50,5 (93)	88,2 (93)	2,4 (166)	2,7 (185)
I	·†	·	·	·	·	·	·
J	55 (±42)	12,5	14,3	100,0 (5)	60,7 (28)	− 1,1 (91)	0 (130)
K	118 (±57)	31,6	29,8	104,5 (88)	55,7 (183)	0,8 (139)	15,1 (139)
L	143 (±109)	4,7	7,7	87,5 (16)	56,3 (16)	− 0,6 (181)	− 1,4 (71)
Mean	·	34,5	24,6	·	·	·	·

* The standard deviation is given in parentheses
† ·: not applicable

TABLE 4 Experiment 1: Total worm count per sheep—the percentage error in total worm burden when aliquots were used to estimate the number of worms that failed to migrate from the agar gel

Group	Mean total worms/sheep*	Percentage error of estimate of total worm burden per sheep					
		Mean error		Maximum error (No. of worms concerned)		Minimum error (No. of worms concerned)	
		1/10 aliquot	2×1/10 aliquots	1/10 aliquot	2×1/10 aliquots	1/10 aliquot	2×1/10 aliquots
A	1 172 (±363)	1,3	1,8	4,0 (1 329)	5,3 (1 216)	−0,1 (1 003)	0 (559)
B	1 674 (±714)	0,6	0,3	5,0 (706)	5,0 (706)	−0,6 (501)	−0,3 (2 284)
C	532 (±270)	2,5	1,4	9,2 (336)	6,3 (336)	0,9 (940)	0,7 (940)
D	974 (±492)	2,5	2,2	10,3 (429)	5,6 (429)	−0,2 (976)	−0,7 (739)
E	1 295 (±512)	4,6	3,2	8,6 (2 001)	7,4 (2 001)	0,6 (1 187)	1,0 (1 187)
F	1 875 (±705)	1,6	1,5	32,4 (37)	32,4 (37)	1,4 (1 962)	1,0 (2 353)
G	41 (± 97)	5,6	2,9	150,0 (6)	133,3 (3)	0 (7)	0 (7)
H	2 231 (±369)	2,2	2,4	7,3 (1 976)	8,9 (1 976)	0,1 (2 792)	0,2 (2 470)
I	2 (± 3)	·†	·	·	·	·	·
J	979 (±547)	0,7	0,8	13,2 (38)	3,1 (739)	0 (883)	0 (883)
K	1 800 (±460)	2,0	1,9	8,6 (1 362)	7,5 (1 362)	0,1 (1 872)	0,6 (912)
L	2 007 (±626)	0,3	0,5	3,2 (444)	2,0 (444)	0,1 (2 423)	0,1 (2 423)
Mean	·	2,17	1,72	·	·	·	·

* The standard deviation is given in parentheses
† ·: not applicable

It is interesting that males of *H. contortus* migrated highly significantly less successfully than females. This phenomenon, the reason for which is obscure, had been noticed previously (Van Wyk, unpublished data, 1977), but in that case the percentages of each sex which failed to migrate were not determined. The rather large bursa of *H. contortus* may be a mechanical hindrance to migration from the agar; on the other hand, the female vulvar flap also appears a likely obstruction to migration.

Cryopreservation did not appear to have any effect on the rate of migration of the Boshof and the O.P.-M. frozen substrains, compared to the Boshof unfrozen substrain.

Although the examination of small aliquots of the agar/ingesta gel for estimating the worms that failed to migrate from the gel seemed to give poor results, the worms were apparently randomly distributed (Appendix) and the effect on the estimate of the total numbers of worms per sheep was very slight, since the mean error was only 2,2% when 1×1/10 aliquot was examined (or 1,7% for 2×1/10 aliquots). When few worms remained in the agar, a higher percentage error was to be expected. This should not, however, cause practical problems as small numbers remaining in the agar mean either that the percentage migration was very high (in which case a relatively inaccurate estimate of the remainder is of little consequence) or that there were very few worms in the animal. In anthelmintic trials the latter usually indicates successful treatment, in which case the relatively large numbers of worms in the untreated controls will make an inaccurate estimate of non-migrant worms in the treated sheep of no practical significance.

EXPERIMENT 2

Experiment 2 was designed:

(1) To examine large-scale worm recovery from both sheep and calves, and

(2) To examine modifications of the techniques of Experiment 1.

Materials and Methods

Experimental animals

Use was made of Dorper sheep and Friesian or Fresian×Jersey calves, raised and maintained worm-free.

Experimental design

The larvae used had been cryopreserved in liquid nitrogen for periods of 28–59 months before being thawed for this trial (Van Wyk & Gerber, 1980 a). The sheep and calves were infested *per os* or by injection into either the duodenum or the abomasum (Tables 6–10).

The animals were fasted and were slaughtered on 2 different days, when the worms were 27–30 days old. The apparent anomaly stems from the fact that the animals were infested on different days with different species of worms.

Worm recovery

The abomasa and intestines were collected as described by Reinecke (1973).

The required volume of warm commercial agar was mixed with gastro-intestinal ingesta to give a final concentration of 0,35% agar and allowed to gel as in Experiment 1. For this procedure 2 concentrations of agar were maintained at 40–50 °C on magnetic stirrers:

(a) A weak solution (0,35% agar) for rinsing specimen bottles (*vide infra*) and

(b) A strong solution (1,4% agar) for mixing with the ingesta at the rate of 1 part agar to 3 parts of ingesta suspension.

Ingesta were washed with saline into 2 ℓ glass jars graduated in 300 mℓ divisions, allowed to sediment, the supernatant poured off for later examination and, if necessary, the remnant was adjusted with saline to the next graduation.

Four hundred mℓ of a mixture of 100 mℓ of strong agar for every 300 mℓ of ingesta was used to fill each frame. The temperature of the suspension after mixing varied between 35 °C and 40 °C.

After the frames had been filled, the jar was rinsed with weak agar solution and the rinsings added to one of the frames. The frames were left to gel at room temperature. The base plates of the frames were removed and the sieves supporting the agar slabs were submerged in basins containing physiological saline at 40 °C. The frames were incubated overnight.

Separation of the ingesta and agar and sieving of the ingesta were carried out as described in Experiment 1.

Worm counts

To assess accurately the agar method of worm recovery, total counts were carried out microscopically on all specimens from each organ (decanted samples, ingesta residues and the worms which migrated from the agar slabs).

Worm identifications

In each specimen the first 50 worms recovered (or 150–200 worms in those that were infested with more than 1 species of worm) were identified, but if fewer than 50 worms (or 150–200) were recovered, all of them were identified.

Results

Percentage migration from agar

The mean percentage migration of 33 916 adults of 5 species of nematodes in sheep infested by inoculation into the abomasum (Table 5) was 95,2% (±5,4), ranging from 90,8% of 15 727 *H. contortus* to 99,6% of 1 714 *M. marshalli.*

In 5 sheep infested by injection into the duodenum (Table 6), 99,0% (±1,3) of 11 296 adult nematodes of 5 species migrated, ranging from 98,5% of 4 550 *N. spathiger* to 100,0% of 2 244 *C. ovina.*

TABLE 5 Experiment 2: Sheep infested by injection of L3 into the abomasum—migration of adult worms from agar (Group A)

Worm species	Sheep: Individual worm burdens*					Total No. of worms		
	1	2	3	4	5	Number	Migration (%)	**Standard deviation (%)
H. contortus								
No. of worms	3 571	4 038	1 489	3 379	3 250	15 727	90,8	8,1
Migration (%)	96,7	90,9	75,2	91,3	90,8			
O. cirmumcincta								
No. of worms	687	539	378	599	502	2 705	97,9	1,5
Migration (%)	99,6	98,1	96,3	96,3	98,6			
M. marshalli								
No. of worms	301	958	—	121	334	1 714	99,6	0,9
Migration (%)	100,0	100,0	—	100,0	98,2			
T. axei								
No. of worms	1 306	1 225	6 172	1 593	999	11 295	99,3	2,1
Migration (%)	99,8	95,1	100,0	100,0	98,1			
T. colubriformis								
No. of worms	496	517	252	610	600	2 475	99,1	0,9
Migration (%)	100,0	100,0	98,8	99,0	97,8			
All species	—	—	—	—	—	33 916	95,2	5,4

* Worms in the agar preparations
** Standard deviation of the percentage of migration, calculated from the worm burdens of individual sheep

TABLE 6 Experiment 2: Sheep infested by injection of L3 into the duodenum—migration of adult** worms from agar (Group B)

Worm species	Sheep: Individual worm bur dens					Total No. of worms		
	a	b	c	d	e	Number	Migration (%)	*Standard deviation (%)
T. falculatus								
No. of worms	1 179	477	142	195	673	2 666	99,1	1,4
Migration (%)	99,9	99,8	99,3	100,0	96,7			
T. colubriformis								
No. of worms	373	343	232	356	206	1 510	99,0	0,9
Migration (%)	100,0	98,3	97,8	99,4	99,0			
N. spathiger								
No. of worms	1 227	774	879	617	1 053	4 550	98,5	2,2
Migration (%)	99,9	99,4	99,3	99,8	94,7			
*O. columbianum***								
No. of worms	126	3	144	14	39	326	99,7	0,4
Migration (%)	99,2	100,0	100,0	100,0	100,0			
*C. ovina***								
No. of worms	419	481	372	437	535	2 244	100,0	0,0
Migration (%)	100,0	100,0	100,0	100,0	100,0			
All species	—	—	—	—	—	11 296	99,0	1,3

* Standard deviation of rate of migration (%), taking the individual worm burdens into consideration
** *O. columbianum*+*C. ovina:* 5th stage worms and not mature adults

TABLE 7 Experiment 2: Sheep infested *per os*—migration of adult** worms from agar (Group C)

Worm species	Sheep: Individual worm burdens					Total No. of worms		
	i	ii	iii	iv	v	Number	Migration (%)	*Standard deviation (%)
H. contortus								
No. of worms	1 008	937	1 451	1 289	1 634	6 319	93,1	1,7
Migration (%)	92,6	96,3	92,2	92,6	92,8			
O. circumcincta								
No. of worms	78	268	392	138	333	1 209	98,3	1,6
Migration (%)	97,4	99,6	96,2	98,6	100,0			
T. axei								
No. of worms	228	554	441	175	338	1 736	99,5	0,4
Migration (%)	100,0	99,6	99,3	100,0	99,1			
T. falculatus								
No. of worms	37	59	115	265	315	791	98,1	3,0
Migration (%)	100,0	100,0	93,0	97,7	99,7			
T. colubriformis								
No. of worms	216	361	323	272	63	1 235	98,5	2,3
Migration (%)	100,0	99,4	99,4	95,6	95,2			
N. spathiger								
No. of worms	273	209	325	355	377	1 539	97,7	0,9
Migration (%)	97,1	97,1	97,5	97,2	99,2			
*O. columbianum***								
No. of worms	207	161	1 165	814	226	2 573	99,6	1,0
Migration (%)	100,0	97,5	99,7	99,9	99,6			
All species	—	—	—	—	—	15 402	96,5	2,5

* Standard deviation of the rate of migration (%), taking the individual worm burdens into consideration
** *O. columbianum:* 5th stage worms and not mature adults

The mean percentage migration of adult worms of 7 species in 5 sheep infested *per os* (Table 7) was 96,5% (±2,5), the range being 93,1% of 6 319 *H. contortus* to 99,6% of 2 573 *O. columbianum*.

The mean migration of 1 983 L4 from the ingesta of 15 sheep was 99,8%; the values for individual species ranged from 99,4% of 172 *N. spathiger* to 100% of 4 *T. falculatus*, 77 *T. axei*, 116 *M. marshalli* and 462 *C. ovina* (Table 8).

While the overall migration of 22 046 adult *H. contortus* from all groups was 91,4%, the migration of 38 568 adults of the other species was 99,0%.

TABLE 8 Experiment 2: Ovine nematodes—migration of L4 from agar

Worm spesies	No. of L4	Migration (%)	Route of infestation
M. marshalli	116	100,0	Abomasum
T. axei	77	100,0	Abomasum+*per os*
T. falculatus	4	100,0	Duodenum
N. spathiger	172	99,4	Duodenum+*per os*
O. columbianum	1 152	99,7	Duodenum+*per os*
C. ovina	462	100,0	Duodenum
All species	1 983	99,8	—

TABLE 9 Experiment 2: Bovine nematodes—migration of L4 from agar

Worm species	Infestation by injection			Infestation *per os*			Total No. of worms (all 4 calves)
	Calf 1	Calf 2	Total (group)	Calf 3	Calf 4	Total (group)	
H. placei adults							
No. of worms	986	1 917	2 903	241	128	369	3 272
Migration (%)	96,5	97,2	97,0	84,2	96,9	88,6	96,0%
O. ostertagi adults							
No. of worms	301	1 590	1 891	241	28	269	2 160
Migration (%)	99,7	99,8	99,8	99,6	96,4	99,3	99,7%
Cooperia spp. adults							
No. of worms	669	0	669	417	2	419	1 088
Migration (%)	91,2	—	91,2	99,5	100,0	99,5	94,4%
N. helvetianus adults							
No. of worms	288	6 829	7 117	204	17	221	7 338
Migration (%)	99,7	99,3	99,3	98,0	100,0	98,2	99,3%
O. radiatum 5ths							
No. of worms	1 299	37	1 336	0	1	1	1 337
Migration (%)	99,8	100,0	99,9	—	100,0	100,0	99,9%
O. radiatum L4							
No. of worms	54	1 805	1 859	2	0	2	1 861
Migration (%)	100,0	100,0	100,0	100,0	—	100,0	100,0%
All species							
No. of worms	—	—	—	—	—	—	17 056
Migration (%)	—	—	—	—	—	—	98,5%

In the 4 calves infested either by injection or *per os* (Table 9), the mean percentage migration of 17 056 L4 and adults of 5 species was 98,5%, the range being 94,4% of 1 088 adult *Cooperia* spp. to 100% of 1 861 L4 *O. radiatum*.

The percentage migrations of all the nematodes recovered from both sheep and calves are summarized in Table 10.

TABLE 10 Experiment 2: Migration of all the nematodes recovered from sheep and calves

Helminths	Migration (%)	Total No. of worms
Sheep		
L4	99,8	1 983
Adults	96,2	60 614
Total worms	96,4	62 597
Calves		
L4	100,0	1 861
Adults	98,3	15 195
Total worms	98,5	17 056

Distribution of nematodes in the various fractions collected

The mean distribution of the adult nematodes of sheep in the various fractions is shown in Table 11: A mean of 3,6% (range 0%–6%) was found in the decanted samples, 21,1% (range 3%–58%) in the mucosal digests and 75,3% (range 42%–96%) in the agar specimens. The corresponding figures for ovine L4 (Table 12) were 7,6% (0%–28%); 40,9% (8%–82%) and 51,5% (0%–90%) and for the calves (Table 13) were 8,0% (0%–23%); 20,2% (4%–42%) and 71,8% (51%–96%). Four genera occur in both sheep and cattle and the percentages of adult worms recovered from the digests of both sheep and calves (Tables 11 & 13) were 8% and 16% for *Haemonchus*, 24% and 42% for *Ostertagia*, 20% and 25% for *Nematodirus* and 3% and 4% for *Oesophagostomum*. In sheep a mean of 9,5% of the relatively larger adult worms (*H. contortus, O. columbianum* and *C. ovina*) was recovered from the digests, compared with 27,2% of the smaller worms (*O. circumcincta, M. marshalli, Trichostrongylus* spp. and *N. spathiger*). For cattle, the respective figures were a mean of 10% for *H. placei*, and *O. radiatum* and a mean of 31% for *O. ostertagi, Cooperia* spp. and *N. helvetianus* (Tables 11 & 13).

TABLE 11 Experiment 2: Ovine nematodes—percentage distribution of adults* in the various fractions

Worm species	Mean distribution (%)			No. of worms
	Decanted samples	Digests	Agar	
H. contortus	6	8	86	25 352
O. circumcincta	6	24	70	6 001
M. marshalli	0	58	42	3 570
T. axei	3	41	56	20 026
T. falculatus	1	12	87	3 867
T. colubriformis	1	8	91	5 883
N. spathiger	2	20	78	8 338
*O. columbianum***	1	3	96	2 991
*C. ovina***	2	7	91	2 645
Mean†	3,6%	21,1%	75,3%	—

* Including young 5th stage worms
** Only 5th stage worms and no adults present
† Mean calculated from the number of worms and not from the percentages listed above

TABLE 12 Experiment 2: Ovine nematodes—percentage distribution of L4 in the various fractions

Worm species	Mean distribution (%)			No. of worms
	Decanted samples	Digests	Agar	
O. circumcincta	18	82	0	45
M. marshalli	0	32	68	171
T. axei	17	57	26	946
T. falculatus	28	36	36	27
T. colubriformis	0	46	54	66
N. spathiger	16	41	43	313
O. columbianum	3	41	56	1 730
C. ovina	2	8	90	482
Mean*	7,6%	40,9%	51,5%	—

* Mean calculated from the number of worms and not from the percentages listed above

TABLE 13 Experiment 2: Bovine nematodes—percentage distribution in the various fractions

Worm species	Mean distribution (%)			No. of worms
	Decanted samples	Digests	Agar	
H. placei adults	1	16	83	3 272
O. ostertagi adults	2	42	56	2 160
Cooperia spp. adults	23	26	51	1 088
N. helvetianus adults	13	25	62	7 338
O. radiatum 5ths	0	4	96	1 337
O. radiatum L4	0	16	84	1 861
Mean*	8,0%	20,2%	71,8%	—

* Mean calculated from the number of worms and not from the percentages listed above

Comment

The modified technique used in this trial was more satisfactory than that employed in Experiment 1 since all volumes of ingesta of both sheep and the young calves could be accommodated easily and their treatment involved less labour than before.

This is apparently the first report of the use of the gelled-agar technique for worm recovery from calves and the results were as good as in sheep. When cases in which bile and/or formalin were added to the agar are excluded, Van Wyk & Gerber (1978) recorded an overall migration of 93,6% of worms in all stages of development from sheep ingesta. Total worm migration was slightly better in the present trial, being 96,4% of 62 597 worms in the sheep and 98,5% of 17 056 worms in the calves (Table 10).

From this table it appears that L4 in sheep ingesta migrated more efficiently than adults. However, the overall difference is largely due to *H. contortus* which migrated less efficiently than all the other species (Tables 5–7) and of which a negligible number of L4 was recovered*. The overall migration of adults,

* Only 1 worm of a total of 22 047 *H. contortus* was in the 4th larval stage, the rest being 5th stage worms or adults; for the purposes of the present publication this single larva was ignored.

TABLE 14 Experiment 2: Percentage migration of adult† worms of those species recovered from more than 1 group of sheep

Worm species	Migration (%)			Range of migration*			
				Minimum		Maximum	
	Group A	Group B	Group C	Migration (%)	No. of worms	Migration (%)	No. of worms
H. contortus	90,8	—	93,1	75,2	1 489	96,7	3 571
O. circumcincta	97,9	—	98,3	96,2	392	100,0	333
T. axei	99,3	—	99,5	95,1	1 225	100,0	175–6 172**
T. colubriformis	99,1	99,0	98,5	95,2	63	100,0	216– 517**
T. falculatus	—	99,1	98,1	93,0	115	100,0	37– 195**
N. spathiger	—	98,5	97,7	94,7	1 053	99,9	1 227
O. columbianum†	—	99,7	99,6	97,5	161	100,0	3– 207**

* Range of individual sheep from all groups combined
** 100% migration was obtained from more than 1 sheep, therefore the range of the individual worm burdens is given
† *O. columbianum:* 5th stage worms and not mature adults

excluding *H. contortus*, was 99,0% of 38 568 worms, whereas that of *H. contortus* was 91,4% of 22 046 worms. In species other than *H. contortus,* only 0,8% more L4 than adult worms migrated.

The variations in migration of those species of worms which were recovered from more than 1 group of sheep are compared in Table 14. There were only very small variations in the percentages of migration between individual worm species recovered from more than 1 group of sheep.

As in sheep, *Haemonchus* migrated less efficiently from calf ingesta than most other genera but *H. placei* migrated more efficiently from calf ingesta than did *H. contortus* from those of sheep. The migration of *O. ostertagi* and *N. helvetianus* adults and *O. radiatum* adults and L4 was probably as effective as can be hoped for from any biological system.

While the migration generally was very satisfactory, a problem encountered in this trial was that large percentages of worms were recovered from the mucosal digest specimens (Table 12–14), the percentage varying from species to species and also within species, according to the stage of development. Furthermore, for worms of the 4 genera occurring in both sheep and calves, higher percentages of each species were found in calf digests than in those from sheep. In addition, fewer of the relatively large adults (e.g. *Haemonchus, Oesophagostomum* and *Chabertia*) were recovered from the digests of both sheep and cattle than smaller genera such as *Ostertagia, Marshallagia, Trichostrongylus, Cooperia* and *Nematodirus.*

Efficient rinsing may probably have been affected by the limitation of volume of saline in this experiment to avoid exceeding the capacity of the bottle in which ingesta from individual animals were collected. The use of more and/or larger containers would obviate the problem, but this would involve more labour. Perhaps with more experience with this modified technique, better results may be expected in future.

Large-scale recovery of these worms could be attempted using the gelled-agar technique. In a small number of trials involving relatively few worms, Van Wyk & Gerber (1978) recovered 96,9% of 961 L4 *O. columbianum,* 90,9% of 24 L3 *O. columbianum* and 81,2% of 32 L3 *H. contortus* by migration from mucosae gelled in agar. However, in a subsequent equally small trial relatively poor results were obtained (Van Wyk, unpublished data, 1979).

In this trial commercial agar at a concentration of 0,35% gave as satisfactory results as those obtained with bacteriological agar at concentrations of 0,65–0,9%. As the cost of commercial agar is 75% cheaper than that of bacteriological agar, a saving of about 85% was realized.

Discussion and Conclusions

These results show that this method may be applied for the recovery of worms from large numbers of sheep and calves, for example, in the non-parametric (NPM) test of Groeneveld & Reinecke (1969), which requires the use of up to 120 sheep to test the efficacy of a single anthelmintic. While the amount of labour is reduced because >90% of the worms are concentrated in the minimum of ingesta and can be easily counted without an overwhelming amount of ingesta having to be searched through, the accuracy of the results is not affected.

In so far as defined accuracy in this NPM test of Groeneveld & Reinecke (1969), as modified by Clark (cited by Reinecke, 1973), is concerned, the obvious problem is the error likely to arise from the estimate of the worms that failed to migrate. This can be important in the NPM in those cases where the classification of the results is not clearly established or where the reduced median count is close to the minimum requirement for any symbol of classification. In these cases, using the standard quantitative procedures, recounts of specified samples are required (Reinecke, 1973) as follows, using standard quantitative procedures: total counts must be done not only on the residues of the median worm count of the controls, but also on those above and below it, and on the highest 1, 2 or 3 values in the treated group.

To facilitate the decision of when additional counts are required, the percentages of error that occur when the total residual worm burdens are estimated from 1, 2, 3 or $4 \times 1/10$ aliquots of ingesta residue, are listed in Table 15. There is a 95% probability that the percentage error (as a percentage of the actual total remaining in the agar) of the estimate will not exceed the listed values if the worms are randomly distributed in the residual agar ingesta when the aliquots are drawn.

TABLE 15 The theoretical errors associated with the use of small aliquots for estimating the residual worms that failed to migrate from the agar gel

Estimated total No. of worms	Error above or below estimated total							
	1/10 aliquot		2×1/10 aliquots		3×1/10 aliquots		4×1/10 aliquots	
	%	No. of worms	%	No. of worms	%	No. of worms	%	No. of worms
15	151,8	23	101,2	15	77,3	12	62,0	9
30	107,4	32	71,6	22	54,7	16	43,8	13
50	83,2	42	55,4	28	42,0	21	33,9	17
70	70,3	49	46,9	35	35,8	25	28,7	20
100	58,8	59	39,2	39	29,9	30	24,0	24
150	48,0	72	32,0	48	24,4	37	19,6	29
200	41,6	83	27,7	55	21,2	42	17,0	34
250	37,2	93	24,8	62	18,9	47	15,2	38
300	33,9	102	22,6	68	17,3	52	13,9	42
350	31,4	110	21,0	74	16,0	56	12,8	45
400	29,4	118	19,6	78	15,0	60	12,0	48
500	26,3	132	17,5	88	13,4	67	10,7	54

It must be noted, however, that when the large-scale application of this agar technique was being investigated, rather poor results were obtained in 2 trials (Van Wyk, unpublished data, 1977).

In the former of these trials (Table 16), very large numbers of worms (a mean of 7 039 *H. contortus* in each of 11 sheep and a mean of 23 320 *T. axei* in 2 of these 11 sheep) were present in the abomasal ingesta and the worms were clumped together, a phenomenon which may have affected the results. But large numbers of the adult worms, which appeared very active when placed in the water-bath, migrated only until they partially protruded from the agar, then became inactive for an unknown reason and later died in the agar. Nevertheless, the poor results applied only to the adult worms, as 94% of the 5th stage worms and L4 of *H. contortus* migrated from the agar. Furthermore, the 2 sheep with the best percentages of migration of *H. contortus* (the main cause of the worm clumps) had the largest numbers of this species. This leaves the cause of these poor results unidentified.

TABLE 16 Unfavourable results in 1 preliminary trial

Worm species	Migration from agar (%)	No. of worms
H. contortus (11)*		
Total worms	80,4	77 428
Adults	78,2**	67 599
5ths	94,5	7 490
L4	99,2	2 339
T. axei (2)*		
Total worms (all adults)	67,7	46 640

* In parentheses: the numbers of sheep involved
** Range: 62,2% of 8 680 worms to 89,6% of 11 480 worms

In the second trial (Table 17) only *H. contortus* gave poor results, the other species migrating at least as well as in the trials described in detail in this paper. Therefore, it seems unlikely that there could have been a problem with the agar preparations.

With this method *H. contortus* and *H. placei* gave poorer results than most other species. Because of the prevalence of *Haemonchus* in the country, factors which may influence their migration from agar (e.g. variation in pH of the agar gel) should be investigated.

TABLE 17 Unfavourable results in the second preliminary trial

Worm species	Migration from agar (%)	No. of worms
H. contortus (7)*	85,7†	3 807 (1)**
O. circumcincta (7)	99,5	1 318 (13)
T. axei (5)	99,3	429 (0)
T. colubriformis (7)	98,8	1 716 (0)
N. spathiger (2)	100,0	4 (4)
O. columbianum (7)	97,4	78 (0)
Trichuris spp. (7)	79,2	77 (0)

* In parentheses: the number of sheep involved
** In parentheses: the number of L4 (included in the total)
† Range: 55,2% of 134 worms to 95,4% of 1 094 worms

One disadvantage of the technique is that it requires large work benches and large water-baths. The amount of space required in the water-bath was reduced by stacking agar frames from the same organ together in each basin. Despite this, in Experiment 2, where total worm recoveries were required from the abomasum as well as from the small and large intestines, up to 10 animals (8 sheep and 2 young calves) only could be handled in a single day, as they required 90 agar frames, 5,5 m² water-bath space (with the water at a depth of 18 cm) and 11 m² bench space (for coagulation of the agar). Frames must be precision-made and base plates tightly-fitted, otherwise preparations would be easily damaged. Furthermore, it is time-consuming to seal the joints with agar before the frames can be filled.

The bench space required could be reduced if the frames of each organ, before being transferred to the water-bath, were stacked on one another as they are filled.

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Appendix

Table 15 above (the theoretically expected errors associated with estimation of the number of worms that remain in the agar by examining small aliquots of the residue in the agar) was calculated on the assumption that the residual worms in the agar are randomly distributed when the aliquots are drawn. This assumption was tested as discussed below.

In Appendix Table 1 the experimental results are ranked and divided into 4 groups according to the actual total number of residual worms that failed to migrate. For each group the mean of the worm burdens as well as of the mean of the variances was calculated.

If the worms are randomly distributed in the agar, the variance of estimates from a 1/10 aliquot will equal $10^2 n(\frac{1}{10})(\frac{9}{10})$, where n is the actual number of worms in the agar (this expression is based on the variance of the binomial distribution which applies to the number of worms in an aliquot). If the mean of the actual total of each of the 4 groups in Table 1 is regarded as n then the second column in Appendix Table 2 can be calculated with the aid of the above formula. The square roots of these variances give the expected standard deviations if the worms were randomly distributed in the agar residue at the time the aliquots were drawn. These are listed in the Column 3.

The mean variances of the 4 groups and the square roots of these variances (i.e. the observed standard deviations) are listed in Columns 5 and 4 respectively.

APPENDIX TABLE 1 Class intervals of the ranked actual worm totals in the agar

1		2		3		4	
Actual total	S.D.*	Actual total	S.D.	Actual total	S.D.	Actual total	S.D.
1	7,1	49	42,4	91	42,4	161	56,6
1	7,1	50	21,2	91	14,1	162	7,1
5	7,1	52	28,3	93	49,5	166	63,6
7	7,1	55	0	97	21,2	167	56,6
8	0	58	56,6	100	84,9	173	7,1
12	0	59	7,1	100	7,1	179	35,4
13	14,1	59	14,1	101	28,3	180	7,1
14	0	60	28,3	101	0	181	14,1
16	28,3	61	28,3	101	7,1	183	21,2
16	7,1	62	7,1	101	35,4	185	0
19	14,1	62	49,5	103	14,1	188	7,1
19	14,1	66	49,5	103	7,1	190	21,2
22	21,2	67	14,1	106	7,1	195	21,2
24	14,1	70	21,2	115	0	197	99,0
26	14,1	71	0	124	14,1	205	113,1
28	7,1	74	7,1	126	42,4	208	35,4
29	28,3	74	35,4	126	28,3	208	42,4
30	21,2	76	14,1	130	14,1	211	106,1
31	7,1	76	28,3	130	42,4	221	7,1
35	7,1	80	42,4	131	21,2	224	28,3
35	14,1	82	42,4	139	28,3	237	70,7
36	0	84	7,1	139	77,8	239	56,6
38	7,1	85	7,1	145	42,4	268	70,7
40	21,2	87	14,1	149	28,3	305	42,4
43	14,1	88	70,7	152	21,2	365	7,1
45	0			156	14,1		
Mean= 28,8	$\overline{var}$**= 176,9 S.D.= 13,30	Mean= 68,3	$\overline{var}$= 996,0 S.D.= 31,56	Mean= 117,3	$\overline{var}$= 1 149,9 S.D.= 33,91	Mean= 207,9	$\overline{var}$= 2 650,2 S.D.= 51,48

* Standard deviation of the estimates from 2×1/10 aliquots

** $\overline{var}$ = mean variance

APPENDIX TABLE 2

n	Theoretical variances	Theoretical standard deviations	Observed variances	Observed standard deviations	Ratio of observed to theoretical variances
29	261,0	16,16	176,9	13,30	0,678
68	612,0	24,74	996,0	31,56	1,627
117	1 053,0	32,45	1 149,9	33,91	1,091
208	1 872,0	43,27	2 650,2	51,48	1,416

To test, for each of the n-values, whether the observed and the theoretical variances differ significantly, the ratio of the 2 variances (the observed divided by the theoretical), multiplied by the number of degrees of freedom of the observed variances can be calculated. These quantities are χ^2-distributed, hence making it possible to use tables of χ^2 distributions to determine the significance.

In Appendix Table 3 the numbers of degrees of freedom, the χ^2 values and the tabulated 5% critical values are listed.

APPENDIX TABLE 3

Degrees of freedom	χ^2 values	5% critical values	
		Lower 2,5%	Upper 2,5%
26	17,63	13,84	41,92
25	40,68	13,12	40,67
26	28,37	13,84	41,92
25	35,40	13,12	40,67

While the observed variance in the second group in Appendix Table 3 (n=68; Appendix Table 2) was only just significantly greater than the theroetical value, no significant differences were found for the other 3 groups. In one case the theoretical value was larger than the observed value and *vice versa* in the other 2. It can be deduced that there are no clear tendencies for the observed variances to differ from those expected in the event of random distrubtion of the worms in the agar ingesta residues and therefore that the assumption of random distribution is not unrealistic.

Statistical appendage to the Appendix

The use of the mean variance of each of the 4 groups as the observed variance for the mean actual worm total can be justified as follows:

Suppose the number of worms per aliquot (X) is binomially distributed. The variance X then equals $n_i pq$, where n_i represents the actual number of worms in the i'th residual agar (sample), p is the size of the aliquot as a fraction of the total volume and q=1-p. The estimated number of worms is $\frac{1}{p}X$ and the variance of this number is

$$\sigma^2_{n_i} = \frac{1}{p^2} n_i pq \quad .$$

For a series of k different values of n_i,

$$\frac{1}{k} \sum_{i=1}^{k} \sigma^2_{n_i} = \frac{1}{p^2} pq \frac{1}{k} \Sigma n_i$$

The left side of this equation is the mean variance and the right side is $\frac{1}{p^2}$ pq multiplied by the mean of the values of n_i, say n.

Therefore: $\sigma^2_n = \frac{1}{p^2} pqn$ The right side therefore equals the variance of the estimated total if the true total equals n. Hence, in Appendix Table 2 the observed variance is calculated after the analogy of σ^2_n, namely as the mean of k observed variances.

If n is reasonably large (≥ 20), the normal distribution with mean $\frac{1}{p}$ np and variance $\frac{1}{p^2}$ npq is a good approximation of the distribution of the estimated number. Under this assumption:

$$p(|\text{True total}—\text{estimated total}| \leq 1,96 \frac{1}{p} \sqrt{npq}) = 0,95$$

In other words, the probability is 95% that the forecast total and the true total will not differ more than

$$1,96 \frac{1}{p} \sqrt{npq} \text{ or } \frac{1,96 \frac{1}{p} \sqrt{npq}}{\text{True total}} \cdot 100\%.$$

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