EVALUATION OF A MODIFICATION OF THE DADA TECHNIQUE FOR THE RECOVERY OF HELMINTH EGGS FROM SOIL, AND DETERMINATION OF THE SPECIFIC GRAVITY OF CERTAIN HELMINTH EGGS USING SUCROSE DENSITY GRADIENT CENTRIFUGATION

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DEDICATION

To my parents

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INTRODUCTION

A number of medically important helminth parasites are acquired through the ingestion of soil contaminated with their infective stages. In certain cases, these are embryonated eggs. In epidemiological studies of infections with such soil-transmitted parasites, it may be desirable to determine the presence and distribution of their eggs in the soil in the community under study. This calls for a technique to separate the eggs from the soil.

In 1979, Dada and Lindquist devised such a technique and reported its recovery efficiency rate to be 67.5% when used for Toxocara canis eggs. The present study explored the possibility of improving the efficiency of this technique via a modification in its methodology. This modification was evaluated for its efficiency in recovering T. canis, Ascaris suum and Trichuris vulpis eggs from artificially-seeded soil.

This study also investigated several helminth eggs in the hope of explaining the different rates of their recovery from soil by differences in their specific gravity.

REVIEW OF LITERATURE

Caldwell and Caldwell (1928) were the first to report on the extraction of helminth eggs from soil. Their method utilized antiformin to release the eggs from the soil particles, sugar solution of high specific gravity to float them, and a small vial to remove them from the surface. They did not furnish any data on its efficiency.

Spindler in 1929 reported on a modification of the Caldwells' technique devised by Cort, Otto and Spindler. It involved treatment of the soil sample

with 30% antiformin for 1 hr with frequent stirrings and subsequent centrifugation in sodium dichromate solution (sp gr 1.35) at 1,000 rpm for 1-2 min. The floated eggs were then looped from the surface using a small vial or the open end of a glass tube. Other than stating that it is possible to recover the "majority of (Ascaris) eggs" using this technique, Spindler did not dwell on its efficiency.

Maplestone and Mukerji (1936) described a technique for the extraction of Ascaris eggs from soil as follows: the sample was treated with 2.13% sodium hydroxide solution for 1-2 hr with frequent stirring, following which chlorine was bubbled through for 10 min. Thirty minutes later, the mixture was centrifuged, the supernatant pipetted off, and saturated salt solution added. Centrifugation was then carried out repeatedly until no more eggs were recovered from the surface ("direct centrifugal flotation pushed to finality"). Using this technique, Maplestone and Mukerji recovered 16.9-51.1% of seeded eggs from moist soil and 2.4-32.4% from dry soil.

In 1942, Stevenson reported on a centrifugal flotation technique utilizing calcium chloride solution (sp gr 1.35-1.40). He stated that $CaCl_2$ gave consistently better results with soil artificially infected with Toxocara canis eggs than zinc sulfate of the same specific gravity or sodium chloride (sp gr 1.20).

Berlinguer (1962) described another method in which he washed the soil sample in NaOH and then centrifuged in sodium nitrate solution (sp gr 1.38). It gave "consistently higher recovery rates (about 95%)" than the methods of Spindler (loc. cit.) and Maplestone and Mukerji (loc. cit.).

Ito and Natsume (1964) used saturated magnesium sulfate solution as a flotation medium and recovered 50% of Ascaris eggs from sandy soil and 10% from clay soil. Treatment with antiformin influenced the results to a greater extent in the case of clay soil.

Lindquist (1966) described a method for isolating Ascaris eggs from soil as follows: the soil sample was passed through 10-, 20- and 40-mesh screens and then 3.5 Gm of the screened soil was mixed with 20 ml of decinormal NaOH. This mixture was poured through a 100-mesh screen into a 100-ml graduated cylinder and the screen flushed with 80 ml more NaOH. The screened mixture was then divided into two 50-ml centrifuge tubes and centrifuged at 1,000 rpm for 1-2 min. The supernatant was decanted and 10 ml of $Na_2Cr_2O_7$ solution added to the sediment in each tube. After thorough shaking, the contents of each tube was transferred to a 15-ml ground-top centrifuge tube, which was trimmed with more $Na_2Cr_2O_7$, topped off with a coverslip, and centrifuged at 1,000 rpm for 1 min. Eggs were then counted off the coverslips. This method recovered only 10-30% of seeded eggs. This low recovery was attributed to the stickiness of the eggs and the repeated screenings.

The foregoing technique was modified by the same worker in 1967 in the following manner: one solution transfer was eliminated, a larger mesh screen but smaller screen area was used, the container was rinsed after each solution transfer, and two flotations were performed on the final sample. Recovery efficiency was thus improved to a large measure: 58-78% of seeded decorticated eggs and 43-66% of seeded intact eggs were recovered.

The World Health Organization Expert Committee on Ascariasis (1967) recommended the following procedure for examination of soil for helminth eggs: to each volume of soil, 2 volumes of bleaching fluid (6% sodium hypochlorite) diluted to 30% were added. The mixture was then allowed to stand for 30 min with frequent stirring, after which it was diluted in 10-20 parts of water, stirred or shaken, allowed to stand just long enough to allow macroscopic particles to settle (for 10-15 sec), then decanted to a clean container. This

supernatant was poured through gauze or a metal strainer, then concentrated by gravity or centrifugal sedimentation. The sediment was then subjected to NaCl flotation. The surface film was poured into a clean tube, diluted, and centrifuged or allowed to settle. The sediment was then examined for eggs.

Borg and Woodruff in 1973 devised a technique to recover Toxocara eggs from the soil. They washed 5 Gm of soil in water by centrifuging in a 50-ml centrifuge tube at 2,000 rpm for 1 min twice. They then added $ZnSO_4$ solution (sp gr 1.18) to the tube, shook it, and let it stand for 10 min, after which they withdrew the supernatant with a plastic syringe and needle. They then passed this through a millipore filter pad, which they examined for eggs.

Dubin et al. (1975) used the Tween 60 method to extract Toxocara eggs from soil. Soil was added to a graduated Erlenmeyer flask to approximately 40 ml volume and enough water was added to bring the volume up to 50 ml. Three drops of Tween 60 (polyoxyethylene sorbitan monostearate) was then added. After shaking for 10 min, the mixture was filtered through gauze into a 15-ml centrifuge tube until 2 ml of filtrate was collected. The tube was then filled with saturated $NaNO_3$ solution and centrifuged at 1,000 rpm for 3 min. More $NaNO_3$ was added to form a positive meniscus, then a slide was applied to the surface and examined for eggs.

Sewell and Urquhart (1976) treated crudely sieved soil with 1% Tween 80 for 1 hr with occasional stirring, then centrifuged the mixture three times. Pooled supernatants were then washed through a sieve of 150u pore size into another of 55u or 32u pore size, which retained the eggs. Sewell and Urquhart did not elaborate on any further treatment or examination of the sample.

Theis et al. (1978) examined soil samples for helminth eggs in the following manner: the sample was soaked in water and then passed through two mesh

screens, a 3.35 mm followed by a 300u standard test sieve. The filtrate was transferred to 50-ml centrifuge tubes and centrifuged at 1,500 rpm for 3 min. The supernatant water was decanted and saturated $ZnSO_4$ solution (sp gr 1.18) or 10% formalin was added and mixed with the sediment. The sample was then centrifuged again, following which aliquots were removed from the surface of the $ZnSO_4$ tube or the bottom of the formalin tube and microscopically examined for eggs.

Quinn et al. (1980) devised the following technique to recover Toxocara and Toxascaris eggs from soil samples. Twenty-five-gram samples were weighed and passed through a coarse sieve to remove stones and grass. To each sample was added 25 ml of 0.0025% Tween 80. The sample was then processed in a homogenizer for 5 min and passed through a second and finer sieve. The residue was washed with a further 25 ml of Tween 80. The sample was then centrifuged at 2,000 g for 10 min. The supernatant was discarded and the precipitate resuspended in flotation solution. Centrifugation was repeated, following which the solution in the tube was topped up to form a positive meniscus. A coverslip was applied, left on for 5 min, and examined. The sediment was processed in a similar manner on three further occasions. In preliminary trials using soil artificially seeded with Toxocara canis eggs, six flotation solutions were evaluated, namely: 33% $ZnSO_4$ (sp gr 1.09), saturated $ZnSO_4$ (sp gr 1.27), 33% $MgSO_4$ (sp gr 1.07), 50% $MgSO_4$ (sp gr 1.14), saturated $MgSO_4$ (sp gr 1.275), and saturated NaCl (sp gr 1.205). These yielded the following recovery percentages, respectively: 1.75, 27.5, 26.75, 72, 82.5, and 51.25. In further studies, the efficiency of saturated $MgSO_4$ solution (which recovered the most eggs in the earlier experiments) was compared with that of saturated $MgSO_4$ solution plus 5% potassium iodide, using less heavily seeded soil (12 eggs/ 25 Gm). The latter

solution consistently recovered more eggs. Accordingly, Quinn et al. used saturated $MgSO_4$ solution plus 5% KI to examine natural soil samples.

Russian workers have been very active in this field of parasitological research. Nizhniak (1977) and Chefranova (1979) reviewed a number of Russian-designed soil helminthological techniques. The basic design of these techniques was similar: eggs were separated from soil particles by mixing in 5% NaOH or water in a special mixer, floated in saturated $NaNO_3$ solution, and recovered by means of a loop or coverslip or by filtration of the supernatant. Isaev et al. in 1969 described a method in which air was blown for 5 min through the soil sample in water in a funnel by means of an air pump attached to the bottom of the funnel. The heavier soil particles were allowed to settle for another 5 min, after which 1-ml aliquots were removed from the suspension with a pipette, preferrably from a depth of 4-5 cm. One hundred percent or nearly 100% positive results were obtained when 4 ml of suspension were examined following artificial contamination of soil with more than 500-1,000 eggs/ 100 Gm of soil, or as little as 1 ml if 5,000 eggs had been added to 100 Gm of soil.

Dada and Lindquist (loc. cit.) devised their technique to use in a survey of soil contamination with Toxocara eggs in Kansas. One gram of soil was weighed and transferred into a 12-ml ground-top centrifuge tube, 9 ml of 0.1N NaOH was added, and the tube was shaken on a mixer for 1 min. The mixture was then centrifuged at 158 g for 5 min. The supernatant was decanted, $ZnSO_4$ solution (sp gr 1.20) was added to the sediment, and the tube was shaken for 2-4 min on the mixer. The tube was then trimmed with more $ZnSO_4$, a coverslip applied, and centrifugation repeated. Five minutes after centrifugation, the coverslip was examined for eggs. The inner wall of the tube was then scraped with a revolving motion with a metal wire and the tube retrimmed with $ZnSO_4$. A coverslip

was applied and centrifugation repeated. Five minutes later, this second coverslip was examined. This process was repeated for a total of five coverslip recoveries. In preliminary trials using soil artificially seeded with 200 T. canis eggs/ Gm, Dada and Lindquist recovered 61.95% of the eggs from ten samples and 67.52% from a second batch of ten, using this technique. [In the actual survey, the technique was modified by substituting 0.1N NaOH with tap water, as "little difference between (the use of 0.1N NaOH) and that of tap water could be detected." Coverslip recoveries were also limited to three, since the preliminary trials indicated that most of the recoverable eggs were retrieved by then.]

The methodology of each of the soil helminthological techniques reviewed above is outlined in Appendix 1. Recovery efficiency when available is also given.

Literature on the specific gravity of helminth eggs is scarce.

Mhaskar in 1923 determined the range of specific gravity of Ancylostoma duodenale eggs using four different solutions of different specific gravities, namely, $MgSO_4$, $CaCl_2$, glycerine, and a 1:1 mixture of saturated $MgSO_4$ and glycerine. He suspended 1 part of egg-positive feces in 40 parts of each solution, allowed the suspension to stand in a test tube for 30 min, then pipetted off the supernatant into another tube. He then added water to each tube to bring the specific gravity close to 1.01, and following centrifugation, counted the eggs in each sediment. In this manner, he found that 7% of the eggs had a specific gravity of 1.03-1.06; 80%, 1.07-1.20; 10%, 1.20-1.29; and 3%, 1.30-1.34.

Sawitz et al. (1929) determined the specific gravity of Necator americanus eggs using $ZnSO_4$ solutions of different concentrations and specific gravities. They put 0.1 ml of fecal suspension in each of six test tubes and filled each

tube to the brim with $ZnSO_4$ of one of the following specific gravities: 1.045, 1.050, 1.055, 1.060, 1.065, and 1.180. They put a coverslip on each tube and centrifuged at 2,640 rpm for 40 sec. They then determined the number of eggs on the surface, in the supernatant, and in the sediment of each tube. The tube with $ZnSO_4$ of 1.055 specific gravity showed the eggs distributed throughout all layers. In solutions of lower specific gravity, most all eggs were seen in the sediment. In higher-specific gravity solutions, most of the eggs floated to the surface. From these results, Sawitz et al. concluded that N. americanus eggs have a specific gravity of 1.055.

Sawitz in 1942 used essentially the same technique to determine the specific gravity of the eggs of five other nematode species. These species and their corresponding specific gravities as determined by Sawitz were: Enterobius vermicularis, 1.115; Trichuris trichiura and T. vulpis, 1.150-1.160; Ancylostoma caninum, 1.055; fertilized Ascaris lumbricoides, 1.110-1.130; and unfertilized A. lumbricoides, 1.20.

Kolomysov in 1978 described a "quicker and simpler" method of measuring the specific gravity of helminth eggs. His technique was basically similar to Mhaskar's (loc. cit.) and Sawitz's (loc. cit.). However, he used four ammonium nitrate solutions of different concentrations and specific gravities. He centrifuged an egg suspension in a solution of a particular specific gravity for 1-1.5 min and examined the surface film. He decanted the supernatant, added a solution of a higher specific gravity to the sediment, centrifuged for 1-1.5 min, and examined the surface film. He then set the lower limit of the range of specific gravity of a particular helminth egg at the specific gravity of that solution in which all the eggs floated to the surface, and the upper limit at the specific gravity of that solution in which all the eggs settled to the

bottom of the tube. In this manner, he determined the following ranges of specific gravity for the eggs of the following helminths: Toxocara mystax, 1.075-1.120; T. canis, 1.070-1.120; Toxascaris leonina, 1.080-1.120; Trichuris vulpis, 1.150-1.185; and Ascaris suum, 1.130-1.180.

In 1961, Marquardt developed a technique to separate Nematodirus spathiger eggs from fecal debris for use in studies on the effect of chemical agents on and the respiration of these eggs. He used a sucrose gradient prepared by layering the following volumes of four dilutions of a standard sucrose solution (sp gr 1.275 at 16 C) in a 50-ml centrifuge tube: 1:2, 8 ml; 1:4, 10 ml; 1:6, 6 ml; and 1:8, 6 ml. He broke the interfaces between the layers using a blunt glass rod and then layered 15 ml of fecal suspension in water over the gradient. He centrifuged the gradient in a rotor with swinging cups at 800 g for 20 min, allowing 5 min for acceleration and 5 for decceleration. In this manner, he obtained a band of N. spathiger eggs between the 1:4 and 1:6 layers and concluded that the specific gravity of these eggs lies between 1.075 and 1.050, the respective specific gravities of these solutions at 16 C. He also reported that when a mixture of several species was centrifuged in a similar gradient, they formed discrete bands. Trichostrongyle (Haemonchus, Ostertagia and Trichostrongylus) eggs turned out to be heavier than N. spathiger eggs, but Nematodirus fillicollis eggs were the heaviest. Coccidian oocysts and Fasciola hepatica, however, were invariably found with the heaviest debris at the bottom of the tube.

Cox (1970) also used sucrose gradients to separate parasitic forms from one another and from debris. He layered 1 ml of the sample on top of an 8-ml, 01.-0.8M sucrose gradient which he then centrifuged at 1,200 g for 1 min. Bacteria settled out at 0.2M, smaller flagellates at 0.3M, larger flagellates at 0.4M, ciliates and opalinids at 0.5M, nematodes at 0.6-0.7M, and helminth eggs

at 0.7M. (The last molar concentration corresponds to approximately 22% sucrose and a specific gravity of 1.0899.) He did not elaborate on the nature or stage of these "nematodes", nor did he clarify the nature of these "helminth eggs".

Magat et al. (1972) determined the specific gravity of unembryonated Ascaris suum eggs of uterine origin and the same eggs following embryonation, using 2-30% and 15-60% sucrose density gradients prepared with a gradient former. Centrifugation was carried out at 125,000 g for 3 hr. The gradients were then allowed to stand overnight at 4 C before refractive indices of samples collected in the area of banding were measured with a refractometer. The average specific gravities were calculated from these refractive indices. These were 1.1036 and 1.0592 for unembryonated and embryonated eggs, respectively. The presence of the eggs (approximately 300,000) did not appreciably alter the refractive index of the sucrose solutions. The difference in specific gravity of unembryonated and embroynated eggs may be related to differences in volume and solids content detected by the same workers.

SECTION I

EVALUATION OF A MODIFICATION OF THE DADA TECHNIQUE FOR THE RECOVERY OF HELMINTH EGGS FROM SOIL

Introduction

Dada and Lindquist (loc. cit.) devised a technique for the recovery of helminth eggs from soil and reported its recovery efficiency rate to be 67.5% for Toxocara canis eggs. In this part of the present study, a modification of this technique was evaluated for its efficiency of recovery of T. canis, Ascaris suum and Trichuris vulpis eggs from artificially-seeded soil.

Materials and Methods

Three experiments were conducted, each involving a different species of egg. Toxocara canis eggs were used in the first experiment, Ascaris suum in the second, and Trichuris vulpis in the third.

Each experiment consisted of two trials. In the first trial in each experiment, decinormal sodium hydroxide was used to wash the soil samples; in the second trial, tap water was used. Twenty samples were run in each trial.

For each experiment, 100 Gm of sterilized clay soil was seeded with approximately 20,000 eggs of the appropriate species. The seeded soil was alternately blended in a laboratory blender and mixed manually with a rubber spatula several times to ensure adequate mixing of the eggs with the soil particles. The seeded soil was then transferred to a Dispo cup (Scientific Products, McGaw Park, Ill.) and stored at 4 C until further use.

Four 1-Gm samples of seeded soil were processed simultaneously as follows: 1 Gm of seeded soil was weighed on a triple-beam balance and transferred into each of four 12-ml ground-top centrifuge tubes. Nine milliliters of 0.1N NaOH (in the first trial in each experiment) or tap water (in the second trial) was added into each tube. The tubes were capped with rubber stoppers and their contents mixed by shaking over a Vortex-Genie mixer (Scientific Products, McGaw Park, Ill.) for 1 min. The stoppers were removed and the tubes centrifuged at 158 g for 5 min. The supernatant was decanted and zinc sulfate solution (sp gr 1.20) was added to about an inch from the top of each tube. The stoppers were replaced and the tubes shaken over the mixer for 1 min. Each stopper was removed and washed into the appropriate tube with $ZnSO_4$. Each tube was then filled to the brim or trimmed with more $ZnSO_4$ and an 18x18-mm #1 coverslip applied to the top. The tubes were centrifuged at 158 g for 5 min. The coverslips were removed onto microscope slides immediately after centrifugation and examined under low power objective. Eggs were counted using a hand tally and this count was recorded as "first coverslip recovery". The supernatant in each tube was decanted into another tube. The second tube was trimmed with $ZnSO_4$, topped with a coverslip, and centrifuged at 158 g for 5 min. After centrifugation, the coverslip was removed and examined and eggs counted. This count was recorded as "second coverslip recovery". The sediment in each of the first set of tubes was resuspended in $ZnSO_4$ by adding $ZnSO_4$ to about an inch from the top of the tube and shaking over the mixer for 1 min. The tube was then trimmed with more $ZnSO_4$, a coverslip applied, and centrifugation carried out at 158 g for 5 min. Examination of the coverslip immediately following centrifugation gave a "third coverslip recovery" egg count. The supernatant and sediment from this centrifugation were processed in a similar manner for "fourth and fifth coverslip recoveries".

For each sample, first, second, third, fourth and fifth coverslip recoveries were totalled to obtain the "total number of eggs recovered". From this was calculated the recovery efficiency rate, as follows:

$$\frac{\text{Total number of eggs recovered}}{200} \times 100 = \text{recovery efficiency rate (\%)}.$$

Data were statistically analyzed by a two-way analysis of variance using the Statistical Analysis System (SAS Institute Inc., Cary, N. C.). Differences between means were considered significant at a $P<0.05$ level.

Results

Results are summarized in Tables 1-4.

The technique was most efficient in recovering Trichuris vulpis eggs (Tables 1 and 2). When decinormal sodium hydroxide was used to wash samples prior to flotation with zinc sulfate, 63.80% of seeded eggs were recovered. Washing with tap water, a significantly greater ($P<0.05$) proportion of eggs, 72.40%, were recovered.

Moderate recovery efficiency was demonstrated for Toxocara canis eggs, 54.95 and 55.83% of these eggs being recovered, using NaOH and tap water, respectively. These means were not statistically different.

The technique showed poor recovery efficiency for Ascaris suum eggs. Only 27.90% of seeded eggs were recovered using NaOH, and even less, 10.60%, using tap water. These means were statistically different ($P<0.05$).

The variability within coverslip recoveries was high but was less marked within total recoveries and recovery efficiency rates (Table 1).

Table 3 shows the percentage of the total egg recovery that was retrieved on each coverslip. Nineteen to 77% of the total egg recovery per trial was recovered on the first coverslip, 19-39% on the second, 4-26% on the third, 0.5-11% on the fourth, and 0.3-5% on the fifth.

TABLE 1. Summary of results on soil egg recovery using the modified Dada technique.[1]

Experimental conditions		Coverslip recovery[2]					Total number of	Recovery
Species	Washing agt	1	2	3	4	5	eggs recovered[3]	efficiency (%)
Toxocara canis	NaOH	69.65 ± 29.44 (22-110)	34.50 ± 24.83 (6-79)	4.50 ± 3.99 (0-16)	0.80 ± 1.24 (0-4)	0.45 ± 0.59 (0-2)	109.90 ± 11.32 (92-128)	54.95 ± 5.66 (46-64)
	tap water	86.05 ± 17.77 (61-119)	21.00 ± 12.38 (4-45)	3.50 ± 2.84 (0-11)	0.50 ± 0.51 (0-1)	0.60 ± 0.75 (0-2)	111.65 ± 15.60 (88-148)	55.83 ± 7.80 (44-74)
Ascaris suum	NaOH	24.20 ± 16.15 (3-63)	15.10 ± 8.15 (5-31)	10.00 ± 7.03 (0-26)	5.85 ± 2.91 (0-10)	0.65 ± 0.88 (0-3)	55.80 ± 16.44 (27-94)	27.90 ± 8.22 (13.5-47)
	tap water	10.00 ± 5.32 (2-22)	5.45 ± 3.59 (0-14)	3.15 ± 3.10 (0-11)	1.55 ± 2.01 (0-7)	1.05 ± 2.46 (0-11)	21.20 ± 7.37 (7-36)	10.60 ± 3.68 (3.5-18)
Trichuris vulpis	NaOH	24.30 ± 19.44 (2-67)	50.15 ± 18.32 (25-108)	33.50 ± 16.61 (6-72)	14.15 ± 7.56 (4-35)	5.50 ± 5.24 (0-20)	127.60 ± 14.82 (105-164)	63.80 ± 7.41 (52.5-82)
	tap water	102.35 ± 23.09 (63-143)	33.85 ± 13.91 (14-62)	6.10 ± 5.23 (0-20)	2.10 ± 2.27 (0-10)	0.40 ± 0.68 (0-2)	144.80 ± 20.30 (103-193)	72.40 ± 10.15 (51.5-96.5)

[1]Twenty 1-Gm samples / species- washing agent combination. Approximately 200 eggs / sample. Results expressed as mean ± standard deviation (range).
[2]Coverslip recovery = number of eggs recovered on a particular coverslip.
[3]Total number of eggs recovered = sum of five coverslip recoveries.

TABLE 2. Mean percentage recovery efficiencies of the modified Dada technique for Toxocara canis, Ascaris suum and Trichuris vulpis eggs using decinormal sodium hydroxide and tap water as washing agents.

Species	Mean recovery efficiency (%)*	
	Washing agent	
	0.1N NaOH	Tap water
Toxocara canis	54.95[a]	55.83[a]
Ascaria suum	27.90[b]	10.60[c]
Trichuris vulpis	63.80[d]	72.40[c]

*n=20. Means with different superscripts are significantly different ($P<0.05$). Least significant difference = 4.66 for comparing any pair of means in the table.

TABLE 3. Percentage of total egg recovery recovered per coverslip.

Experimental conditions		Percentage of total egg recovery recovered per coverslip				
Species	Washing agt	1	2	3	4	5
Toxocara canis	NaOH	63.38	31.39	4.09	0.73	0.41
	Tap water	77.07	18.81	3.13	0.45	0.54
Ascaris suum	NaOH	43.37	27.06	17.92	10.49	1.16
	Tap water	47.17	25.17	14.86	7.31	4.95
Trichuris vulpis	NaOH	19.04	39.31	26.25	11.09	4.31
	Tap water	70.68	23.38	4.21	1.45	0.28

Table 4 shows the cumulative percentage of the total egg recovery retrieved per coverslip. At least 95% of all the eggs recovered were retrieved on the first three coverslips in the case of Toxocara eggs, the first four in the case of Ascaris eggs, and the first four and first three, using NaOH and tap water, respectively, in the case of Trichuris eggs. Three coverslips recovered at least 85% of all recoverable eggs in all trials.

Foaming was observed when NaOH was used to wash samples. This was particularly marked on the first coverslip, rendering examination difficult, but diminished with succeeding coverslips. This phenomenon was also observed to a greater extent in the Trichuris trial. It was not observed when tap water was used.

Discussion

The main difference between the Dada technique and the modification herein reported and evaluated was the manner of treatment of the sample following the first coverslip recovery. In the Dada technique the inner wall of the centrifuge tube was scraped with a metal wire to dislodge eggs that might be adherent. In the modification the supernatant was decanted into another tube which was then trimmed with more zinc sulfate, topped with a coverslip, and centrifuged for the second coverslip recovery. It was thought that in this manner, eggs that had not reached the surface and were still in the supernatant at the time of the first coverslip recovery would be recovered. Furthermore, the sediment in the first tube was resuspended in $ZnSO_4$ and the tube retrimmed, topped with a coverslip, and centrifuged for the third coverslip recovery. The rationale behind this step was that eggs that might have been trapped in soil particles and brought down to the sediment with them or those that might be sticking to

TABLE 4. Cumulative percentage of total egg recovery recovered per coverslip.

Experimental conditions		Cumulative percentage of total egg recovery recovered per coverslip				
Species	Washing agt	1	2	3	4	5
Toxocara canis	NaOH	63.38	94.77	98.86	99.59	100
	Tap water	77.07	95.88	99.01	99.46	100
Ascaris suum	NaOH	43.37	70.43	88.35	98.84	100
	Tap water	47.17	72.88	87.74	95.05	100
Trichuris vulpis	NaOH	19.04	58.35	84.60	95.69	100
	Tap water	70.68	94.06	98.27	99.72	100

the sides of the tube would be recovered. The supernatant and sediment from this third centrifugation were similarly processed for fourth and fifth coverslip recoveries. In the Dada technique, five coverslip recoveries were obtained, each one (excluding the first) following scraping of the inner wall of the tube, retrimming of the tube with $ZnSO_4$, and recentrifugation.

The results herein presented indicate that the aforementioned steps in the modified technique did increase egg recovery. As seen in Table 3, 19-39% of the total egg recovery per trial was retrieved from the first supernatant (or on the second coverslip), 4-26% from the first sediment (or on the third coverslip), up to 11% from the second supernatant (or on the fourth coverslip), and up to 5% from the second sediment (or on the fifth coverslip).

The efficiency of the modified technique cannot be justly compared to the original technique, as runs using the latter were not performed. It is a fair assumption, however, that, at best, the original technique would have recovered only as many eggs as were recovered on the first and second coverslips in the modified technique. All eggs that for any reason settled in the sediment during the first centrifugation would have been lost because the sediment would not have been resuspended, but in fact more and more compacted with each centrifugation.

The three species of eggs used in this part of the study were chosen on the basis of their public health significance and the potential for practical application of the technique for their recovery from soil. The public health importance of Toxocara canis as the causative agent of visceral larva migrans in man is well-recognized. While the pathogenicity of Ascaris suum and Trichuris vulpis in man is less established, human infections with these species have been reported (Crewe and Smith, 1971; Kenney and Yermakov, 1980). In

addition, Ascaris lumbricoides, being morphologically similar to A. suum, may be expected to behave in a similar manner as A. suum did in this experiment.

The recovery efficiency of the modified Dada technique was found to vary with the species of egg involved. It recovered significantly more Trichuris eggs ($P<0.05$) than Toxocara eggs, and significantly more Toxocara eggs ($P<0.05$) than Ascaris eggs. The reason or reasons for this observation are open to conjecture. A very likely explanation, however, is the difference in the shell structure of these eggs. While the trichurid egg is smooth-shelled, the surface of the ascarid egg is a network of depressions and ridges (Ubelaker and Allison, 1975) that may serve as "handholds" for soil particles. The pits are wider in Ascaris eggs than in Toxocara eggs. Conceivably, bigger and heavier soil particles are caught in these wider pits to bring the eggs down with them to the sediment. In addition, Ascaris eggs are known to be "sticky" (Fairbairn, 1957). Whether or not the same is true for Trichuris and Toxocara eggs is not known, but this may be partly responsible for the low Ascaris recoveries.

Another possible explanation for the results obtained is a difference in specific gravity of the eggs. This point was pursued further in the second part of this study.

A third possibility that was entertained was that of a difference in surface charge of the eggs. It may be that Ascaris eggs are more electrically attracted and more strongly bound to soil particles than Trichuris or Toxocara eggs and therefore more difficult to dissociate from soil particles. This point requires further investigation.

Another interesting observation was that the recovery efficiency of the technique for a particular species depended to a large extent on the manner of treatment of the sample prior to $ZnSO_4$ flotation. A significantly higher

recovery rate ($P<0.05$) was obtained for Trichuris eggs when the sample was washed with tap water than when sodium hydroxide was used. This was probably due to the extensive foam production observed with the use of NaOH, most marked on the first coverslip, which might easily have led to some eggs being missed during examination. This foaming might have been the result of a reaction between the residual NaOH in the washed sample and the $ZnSO_4$, because, as the run proceeded and the NaOH was presumably washed off during the repeated centrifugations in $ZnSO_4$, the foaming diminished. Besides, foaming was not observed when tap water was used. It may also be due to a reaction between the formalin in the egg suspension and the NaOH. Still another likelihood is that this foam represented air that was trapped in the soil, but if this were the case, this should have diminished after the NaOH wash. Besides, as noted earlier, the phenomenon was not observed when tap water was used. For some inapparent reason, foaming was observed to a lesser degree in the Toxocara and Ascaris trials. It must be emphasized that the type of soil used was the same through all trials. Dada and Lindquist (loc. cit.) did not report the occurrence of foaming in their experiments.

Significantly higher Ascaris recoveries ($P<0.05$) were observed when NaOH was used to wash the samples than when tap water was used. This may be attributed to the dissociating effect of NaOH on the eggs and soil particles.

Recovery efficiency of the technique for Toxocara eggs was not significantly affected by the preflotation treatment of the sample, this being essentially the same whether NaOH or tap water was used.

In summation, NaOH should be used for optimum results. However, further studies are indicated to investigate the foaming phenomenon associated with the use of NaOH in this technique and ways to prevent it. One possible solution to

the problem is to wash the sample in distilled water prior to $ZnSO_4$ flotation to remove any residual NaOH.

As seen in Table 4, at least 95% of all the eggs the technique is capable of recovering can be retrieved on the first three or four coverslips depending on the species involved and/or washing agent used. Three coverslips will retrieve at least 85% of all recoverable eggs in any case. It must be pointed out, however, that "centrifugal flotation pushed to finality", i.e., repeated centrifugation until no more eggs can be recovered, as was done by Maplestone and Mukerji (loc. cit.), was not done in these experiments, so more eggs might very well have been recovered after the fifth coverslip in certain cases.

One variable that needs further investigation is that of flotation solution. The same technique may be evaluated using flotation solutions other than $ZnSO_4$. Saturated $MgSO_4$ (sp gr 1.275) used by Quinn et al. (loc. cit.) is particularly promising.

A second variable that may be improved upon is the length of exposure of the sample to NaOH. A longer exposure time than the one minute employed in the present study may lead to more eggs being dissociated from soil particles and recovered.

The single most significant variable in this study was the actual number of eggs available for recovery in each sample. It can only be assumed that, given the relatively consistent egg counts obtained on the egg suspensions and the thorough mixing of the soil samples with the eggs, the actual number of eggs in each 1-Gm sample was at least close to 200. Obviously, the best way to reduce or eliminate sampling error is to seed individual 1-Gm soil samples with a precise number of eggs, i.e., eggs that have actually been enumerated. This may be difficult to accomplish. The eggs may be washed off from the slide on

which they were counted directly into the soil sample. Some may be expected to stick to the slide, but these may be counted and their number subtracted from the original count. A second alternative is to determine the concentration of the egg suspension in the manner employed in this study, but to seed individual 1-Gm samples with smaller aliquots of the suspension. In this way, the error involved in seeding a large batch of soil and then taking small samples from this batch for processing is eliminated, and the necessity of ensuring an even distribution of the seeded eggs in the larger batch is removed.

SECTION II

DETERMINATION OF THE SPECIFIC GRAVITY OF CERTAIN HELMINTH EGGS USING SUCROSE DENSITY GRADIENT CENTRIFUGATION

Introduction

Since the principle underlying any flotation technique is the difference in specific gravity of the helminth eggs and flotation solution, it was thought that differences in the specific gravity of different species of helminth eggs might account for the different rates of recovery of these eggs from soil as observed in the previous experiments, such that more of the lighter eggs might be recovered by centrifugation in a high-specific gravity solution.

As was demonstrated by Marquardt (loc. cit.) and Cox (loc. cit.), it is possible to separate helminth eggs from other particles in a given suspension by centrifugation in a density gradient column encompassing their density, such that they ultimately reach equilibrium positions in the gradient, i.e., positions at levels of the gradient corresponding to their own densities. This technique is known as density gradient centrifugation.

In this part of the study, sucrose density gradient centrifugation was employed to isolate several species of helminth eggs, following which the refractive index at the level of the gradient at which they settled was measured. The corresponding specific gravity was then taken as the specific gravity of the respective helminth eggs.

Materials and Methods

A total of ten species of helminth eggs were studied. These were *Toxocara canis*, *Toxascaris leonina*, *Ancylostoma caninum*, *Trichuris vulpis*, *Physaloptera* sp, *Taenia* sp, *Toxocara cati* (embryonated), *Ascaris suum*, *Trichuris suis*, and *Parascaris equorum*.

Sucrose gradients were prepared as follows. The following sucrose solutions were prepared: 3% (sp gr 1.0099), 13% (sp gr 1.0507), 24% (sp gr 1.0990), 35% (sp gr 1.1513), and 54% (sp gr 1.2518). Two milliliters of each solution was carefully layered by handpipetting in turn from the heaviest (54%) to the lightest (3%) in a 15-ml graduated centrifuge tube using a graduated pipette. The tube was then capped with a rubber stopper and stored at 4 C for approximately 48 hr to eliminate the sharp interfaces between the layers and establish a smooth gradient. (This was checked by measuring the refractive index of 0.02-ml samples drawn at every ml graduation on the centrifuge tubes from four gradients 48 hr after preparation. A graph was plotted from the average of the corresponding specific gravities to show the specific gravity slope of such gradients 48 hr after preparation. This graph is shown in Appendix 2.)

Fecal eggs were used in all cases. In some cases, fecal concentrates were used, in others, egg concentrates. In either case, the suspending medium was distilled water. Two milliliters of fecal or egg concentrate was layered over the gradient.

The gradient was then centrifuged at 800 g for 20 min. To prevent jarring or turbulence in the gradient, acceleration and deceleration each utilized a 5-min period.

Following centrifugation, the gradient was carefully removed from the centrifuge and examined. The egg band was located and its span determined.

(In preliminary experiments, samples were pipetted off from all visible "bands" and examined microscopically for the presence of eggs. Only those bands from which eggs were recovered were further analyzed.) When no band was evident, the eggs were located by pipetting off a sample at every 0.5-ml graduation on the centrifuge tube and examining these samples microscopically.

A sample was then taken from the band for refractive index measurement. The level from which this sample was taken depended on the width of the band. If the band was narrow, it was taken from the middle of the band. In the case of wide bands, it was taken from the level at which the concentration of eggs was highest. This level was determined by drawing a 0.05-ml sample using an Eppendorf pipette (Brinkmann Instruments, Inc., Westbury, N. Y.) at every 0.1-ml graduation on the centrifuge tube within a span of the gradient that included the band, and counting the eggs in each sample. Similarly, when no band was formed, a 0.05-ml sample was drawn using an Eppendorf pipette at every 0.1-ml graduation on the centrifuge tube within a span of the gradient that included all levels that were found positive for eggs in the preliminary experiment described in the preceding paragraph. The eggs in each sample were counted and the sample for refractive index measurement drawn from the level of the gradient that yielded the highest egg count.

To collect the sample for refractive index measurement, the gradient was carefully drawn down to the desired level using an aspirator. A Pasteur pipette was then used to draw off the sample from the surface. Two to 3 drops were collected. In later experiments, an Eppendorf pipette was used to collect 0.02-ml samples.

Refractive index was measured on a Bausch and Lomb Abbe-3L refractometer (Bausch and Lomb, Rochester, N. Y.). This was converted to specific gravity

using an interpolation table prepared from standard values as set forth by Weast (1980). Appropriate corrections were made using a standard correction table (Weast, loc. cit.) when readings were made at temperatures other than the prescribed 20 C.

Four gradients were run for each species of egg. In some instances, all four were used to measure the refractive index, but in most cases, one gradient was used to locate the highest concentration of eggs in the gradient and the remaining three to measure the refractive index at that level.

Results

Results are summarized in Table 5.

In all cases, eggs of a particular species formed identical bands in the four gradients run for that species.

Toxascaris leonina and Ancylostoma caninum turned out to be the lightest of the ten species studied. Eggs of these species were present in the same sample and formed a single band that spanned from 6.7 to 7.0 (these figures and similar ones to follow refer to ml graduations on the centrifuge tubes). Microscopic examination of 0.05-ml samples drawn at every 0.1-ml graduation from 6.0 to 7.5 showed them to be present in appreciable numbers from 6.6 to 7.3. The highest concentration, however, was found at the 7.1 level, at which the specific gravity ranged from 1.0549 to 1.0573 as measured on three gradients and averaged 1.0559.

Toxocara canis eggs settled in a narrow band from 5.3 to 5.5 (Figure 1). Refractive index was measured on samples taken from the 5.4 level. Specific gravity at this level as measured on four gradients ranged from 1.0891 to 1.0910 and averaged 1.0900.

TABLE 5. Specific gravity of certain helminth eggs as determined using sucrose density gradient centrifugation.

Species[1]	Span of band formed[2]	Sampling level[4]	Specific gravity[5]
Toxascaris leonina[a]	6.7-7.0	7.1	1.0559
Ancylostoma caninum[a]	6.7-7.0	7.1	1.0559
Toxocara canis	5.3-5.5	5.4	1.0900
Parascaris equorum	4.6-5.4	5.3	1.0969
Toxocara cati	4.7-5.2	5.1	1.1005
Ascaris suum[b]	3.6-4.2	4.0	1.1299
Trichuris suis[b]	3.6-4.2	4.0	1.1299
Trichuris vulpis	2.9-3.7	3.5	1.1453
Taenia sp	NVB[3]	1.6	1.2251
Physaloptera sp	NVB	1.3	1.2376

[1]Species with identical superscripts were present in the same sample and formed a single band. Both species were found in the highest concentration at the same level of the gradient.

[2]Figures indicate the spans of the bands formed in terms of the ml graduations on the centrifuge tubes.

[3]NVB = no visible band formed.

[4]Sampling level was either the middle of the band, in the case of narrow bands, or the level of the gradient at which the concentration of eggs was highest, in the case of wide bands or when no band was formed.

[5]Average of specific gravities extrapolated from refractive indices at sampling level as measured on three or four gradients.

Parascaris equorum eggs settled just below the level of the T. canis egg band, forming a wide band from 4.6 to 5.4. Samples taken from every 0.1-ml graduation from 4.1 to 6.0 were all positive for eggs, but these were most numerous at the 5.3 level. Specific gravity at this level as measured on three gradients ranged from 1.0968 to 1.0971 and averaged 1.0969.

Embryonated Toxocara cati eggs formed a band that approximated that of P. equorum eggs. This spanned from 4.7 to 5.2 (Figure 2). Sampling for egg counts was done from 4.5 to 5.5, all of which levels were positive for eggs, but the highest concentration was located at the 5.1 and 5.2 levels. Sampling for refractive index measurements was done from the 5.1 level of three gradients. Corresponding specific gravities ranged from 1.1004 to 1.1006, averaging 1.1005.

Ascaris suum and Trichuris suis eggs were present in the same sample and formed a single band that spanned from 3.6 to 4.2 (Figure 3). Microscopic examination of 0.05-ml samples from every 0.1-ml graduation within this span showed both species to be most numerous at the 4.0 level. Specific gravities based on refractive indices of samples from this level in three gradients ranged from 1.1283 to 1.1310 and averaged 1.1299.

Trichuris vulpis eggs turned out to be heavier than the species in swine. These eggs settled over a wide span of the gradient, from 2.9 to 3.7. Grossly, they did not seem to be uniformly distributed over this span, and this was confirmed by microscopic examination of 0.05-ml samples drawn from every 0.0-ml graduation from 2.5 to 4.0. However, the greatest number settled at the 3.5 level, at which the specific gravity ranged from 1.1441 to 1.1468 as measured on three gradients and averaged 1.1453.

The heaviest species studied were Taenia sp and Physaloptera sp. These eggs did not form a visible band, but microscopic examination of samples drawn

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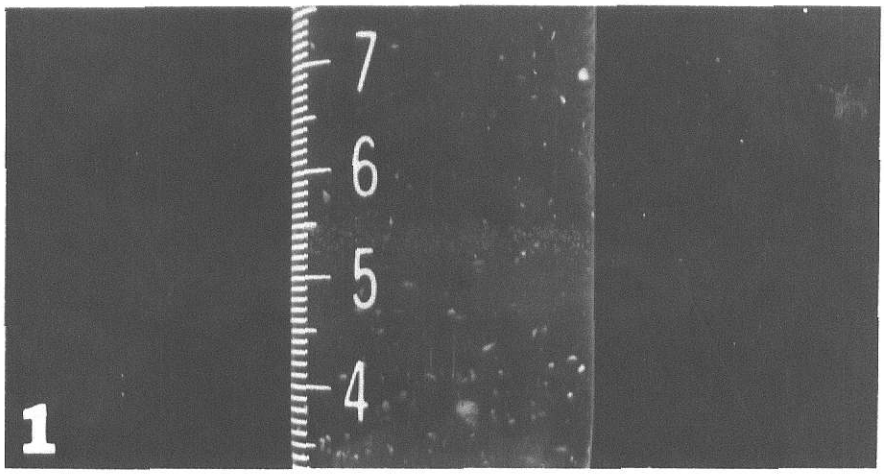
Figure 1. *Toxocara canis* egg band.

Figure 2. *Toxocara cati* (embryonated) egg band.

Figure 3. *Ascaris suum* - *Trichuris suis* egg band.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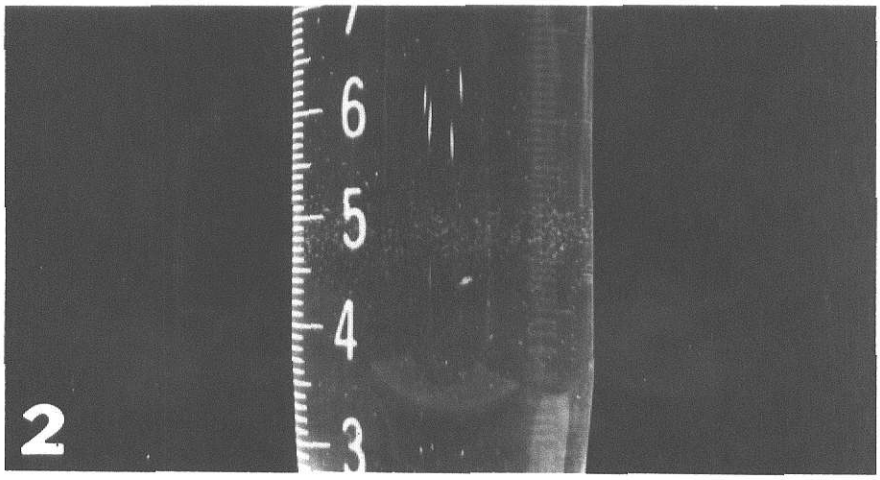
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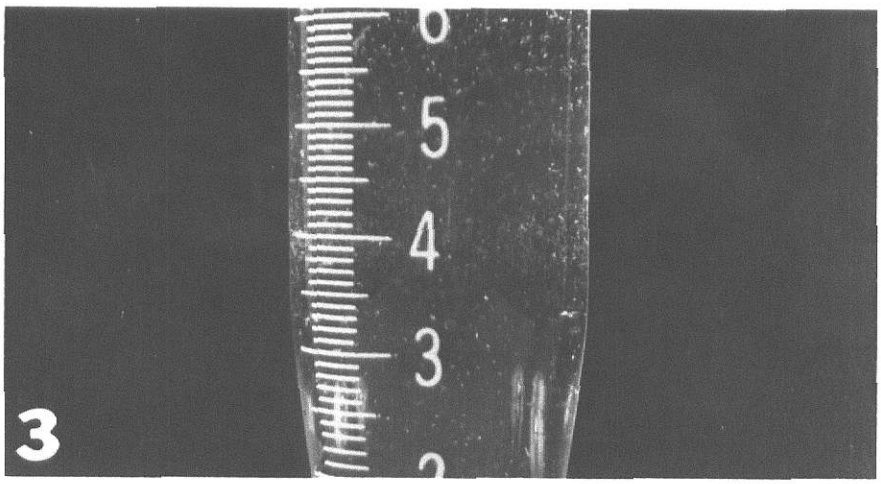
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at 0.5-ml intervals along the gradient showed them to be present in the lower levels (3.5 and below). Further analysis of these levels on another gradient showed the highest concentration of these eggs to be at the 1.6 level. However, while Taenia sp eggs progressively diminished in number at levels lower than this, Physaloptera sp eggs did not. The lowest level of the gradient analyzed, 1.0, still contained an appreciable number of Physaloptera sp eggs. Thus, while refractive index measurements for Taenia sp were made at the 1.6 level, these were made at 1.3 level in the case of Physaloptera sp. Specific gravity at the 1.6 level ranged from 1.2244 to 1.2257 and averaged 1.2251, and at the 1.3 level ranged from 1.2372 to 1.2380 and averaged 1.2376.

In many instances, some eggs were seen in the sediment, when this was examined.

Discussion

This part of the study was prompted by the varying results obtained with different species of eggs in the preceding experiments. It was thought that these observations could be explained by differences in the specific gravity of the eggs, such that the lighter eggs would be recovered in greater numbers than the heavier eggs.

Differences in specific gravity were indeed demonstrated among the ten species of eggs studied. Specific gravities ranged from 1.0559 (for Toxascaris leonina and Ancylostoma caninum) to 1.2376 (for Physaloptera sp). However, it was found that the Trichuris vulpis egg has a higher specific gravity than the Toxocara canis or Ascaris suum egg. Therefore, factors other than specific gravity differences must be responsible for the higher recoveries of T. vulpis eggs in the previous experiments.

Similarities in specific gravity were also observed. These were between T. leonina and A. caninum, and between A. suum and Trichuris suis, both with a specific gravity of 1.1299.

It must be emphasized that the values reported represent the specific gravities of the majority of the eggs of the different species. However, as sampling from different levels of the gradient revealed in most instances, many eggs settled at levels above and below the sampling level. In some cases, eggs were found in the sediment. This indicates that there is no one specific gravity for any one species of egg. Rather, this consists of a range of values. This was earlier observed and reported by Mhaskar (loc. cit.) and Kolomysov (loc. cit.). This is probably due for the most part to individual variation. Differences in "age" or stage of development may also be a contributing factor. Further studies are in order to determine the precise specific gravity range of these eggs. More meaningful information may also be derived from running a known number of eggs through a gradient and determining the percentage distribution of these eggs throughout the gradient.

Some of the specific gravities reported are estimated in the fourth decimal place on account of the corrections that had to be made to account for the temperatures at which the refractive index measurements were made, which were 1-4 degrees higher than the prescribed 20 C. These corrections involved converting the refractive index measured to percentage of sucrose, correcting the percentage of sucrose according to a standard correction table, and converting the corrected percentage of sucrose to specific gravity. The estimation occurred in the last step, as the interpolation table that was prepared did not have the corresponding specific gravity for every percentage of sucrose to four decimal places.

All eggs were tested in the stage in which they were passed in the feces except the Toxocara cati eggs, which were embryonated. Kolomysov (loc. cit.) reported that the specific gravity of unembryonated T. cati eggs ranges from 1.075 to 1.1200. Whether embryonated T. cati eggs have the same range of specific gravity remains to be seen. However, Magat et al. (loc. cit.) reported a decrease in the specific gravity of A. suum eggs following embryonation. This was associated with a decrease in volume and solids content. The results of the present study are further compared with previous reports in Appendix 3.

The error involved in sampling for egg counts and refractive index measurements must be recognized. Samples were drawn by handpipetting, and it is conceivable that the slightest vertical movement of the tip of the pipette could make a difference, however insignificant, in the resulting egg count or refractive index measurement, as the gradients as prepared were quite steep (Appendix 2). However, within this limitation, as much care as possible was exercised in collecting the samples.

Notwithstanding, the technique herein reported is still more precise than those of Mhaskar (loc. cit.), Sawitz et al. (loc. cit.), or Kolomysov (loc. cit.), all of which estimated the specific gravities or specific gravity ranges of eggs from their behavior in a small series of flotation solutions of different concentrations and specific gravities. However, the present technique can still stand refinement. For instance, a gradient former may be used to prepare the gradients. This will ensure uniformity of the gradients, although the manually-prepared gradients used in this study proved to be entirely satisfactory, bands formed by one species of egg in three or four gradients being identical. A gradient former will also save time, as gradients are ready for use immediately after preparation.

The specific gravity range of the gradient used in this study was adequate for all of the eggs studied except the Taenia sp and Physaloptera sp eggs, whose specific gravity ranges apparently go beyond the upper limit of the gradient. For such eggs, the gradient may be modified by eliminating the lowest concentration of sucrose (3%) and incorporating a heavier solution, possibly one in the 70-80% range.

In preliminary trials, gradients were occasionally stored at refrigeration temperature for at least 24 hr following centrifugation with eggs. In such cases, the eggs remained in the same position in the gradient, i.e., the bands they formed were stationary. This indicates that suspension in the gradient did not alter their specific gravity (otherwise the bands would have shifted), and also that the centrifugation time employed was adequate for the eggs to reach equilibrium levels in the gradient (otherwise the bands would have moved to a lower level in the gradient).

One interesting observation was that the heaviest species among the ten studied were also the smallest, Taenia sp and Physaloptera sp. The high specific gravities of these species, 1.2251 and 1.2376, respectively, may very well explain the relative infrequency with which these infections are detected. In addition, Taenia sp was found in appreciable numbers in the sediment. Physaloptera sp may also have been present in the sediment, but these small, colorless eggs were more difficult to pick out in the debris than the dark-brown Taenia sp eggs.

The aforementioned species were also the ones that failed to form visible bands. This may be due to the small size of these eggs coupled with the presence of pigment and debris at the levels they settled in. In the case of Physaloptera sp, an additional factor may be the low number of eggs in the concentrate.

Specific gravity is defined as the ratio of the mass of a body to the mass of an equal volume of water at 4 C or other specified temperature (Weast, loc. cit.). Thus, the specific gravity of an egg depends largely on its volume and mass (solids and water content). Therefore, while Physaloptera sp and Taenia sp eggs are much smaller than hookworm or ascarid eggs, they actually have higher specific gravities, because their mass per unit volume is greater, there being essentially no perivitelline space in these eggs.

Notwithstanding the failure to explain the results of the previous soil experiments in terms of specific gravity differences of the eggs, the results obtained in this part of the study may be used to select the appropriate concentration of a particular flotation solution to use for a particular species of egg. The technique described may also be utilized to prepare egg concentrates when a minimum of extraneous debris is desirable, since only debris with the same specific gravity as that of the eggs should settle at the same level of the gradient.

SUMMARY AND CONCLUSIONS

Section I

The efficiency of the modified Dada technique for the recovery of helminth eggs from soil was dependent on the species of egg involved and the washing agent used. The technique had good, moderate and poor recovery efficiency for Trichuris vulpis, Toxocara canis and Ascaris suum eggs, respectively. More T. vulpis eggs were recovered using tap water, but more A. suum eggs were recovered using decinormal sodium hydroxide. The washing agent used did not affect T. canis egg recovery.

At least 95% of all recoverable eggs were retrieved on the first three or four coverslips depending on the species involved and the washing agent used. Three coverslips recovered at least 85% of all recoverable eggs in any case.

Section II

The specific gravities of the following helminth eggs as measured by a technique employing sucrose density gradient centrifugation were: Toxascaris leonina and Ancylostoma caninum, 1.0559; Toxocara canis, 1.0900; Parascaris equorum, 1.0969; Toxocara cati (embryonated), 1.1005; Ascaris suum and Trichuris suis, 1.1299; Trichuris vulpis, 1.1453; Taenia sp, 1.2251; and Physaloptera sp, 1.2376.

These determinations agreed with or approximated those of previous workers. The specific gravities of P. equorum, T. suis, Taenia sp and Physaloptera sp eggs are reported for the first time.

APPENDIX 1. Summary of soil helminthological methods.

Worker/s	Washing agent	Flotation solution	Manner of egg recovery	Other treatments	Recovery efficiency[1]
Caldwell and Caldwell (1928)	antiformin	high - sp gr sugar solution	eggs looped from surface	-	ND[2]
Spindler (1929)	30% antiformin	$Na_2Cr_2O_7$ (sp gr 1.35)	eggs looped from surface	-	ND
Maplestone and Mukerji (1936	2.13% NaOH, chlorine bubbled through	saturated salt solution	eggs recovered on coverslips	-	16.9-15.1% (moist soil), 2.4-32.4% (dry soil) [Ascaris]
Stevenson (1942)	-	$CaCl_2$ (sp gr 1.35-1.40)	ND	air trapped in soil removed by vacuum pump	ND
Vasilkov and Gefter (1948) (from Chefranova, 1979)	5% NaOH	saturated $NaNO_3$	eggs looped from surface	sample mixed with NaOH in Shuttel's mixer	44.6%
Namitokov (1961) (from Chefranova, 1979)	5% NaOH	saturated $NaNO_3$	supernatant filtered	sample mixed with NaOH in Shuttel's mixer	60%

APPENDIX 1. (Continued)

Worker/s	Washing agent	Flotation solution	Manner of egg recovery	Other treatments	Recovery efficiency[1]
Gefter and Shigina (1962) (from Chefranova, 1979)	5% NaOH	saturated $NaNO_3$	eggs recovered on coverslip	sample mixed with NaOH in Shuttel's mixer	"twice more effective than Vasilkov and Gefter's (1948)"
Berlinguer (1962)	NaOH	$NaNO_3$ (sp gr 1.38)	ND	-	"higher recovery rates (about 95%)" than obtained by Spindler or Maplestone and Mukerji
Ito and Natsume (1964)	antiformin	saturated $MgSO_4$	ND	-	50% (sandy soil), 10% (clay soil) [Ascaris]
Lindquist (1966)	0.1N NaOH	$Na_2Cr_2O_7$	eggs recovered on coverslips	sample screened prior to flotation	10-30% [Ascaris]
Lindquist (1967)	0.1N NaOH	$Na_2Cr_2O_7$	eggs recovered on coverslips	sample screened prior to flotation, flotation repeated	57-78% (decorticated eggs), 43-66% (intact eggs) [Ascaris]

APPENDIX 1. (Continued)

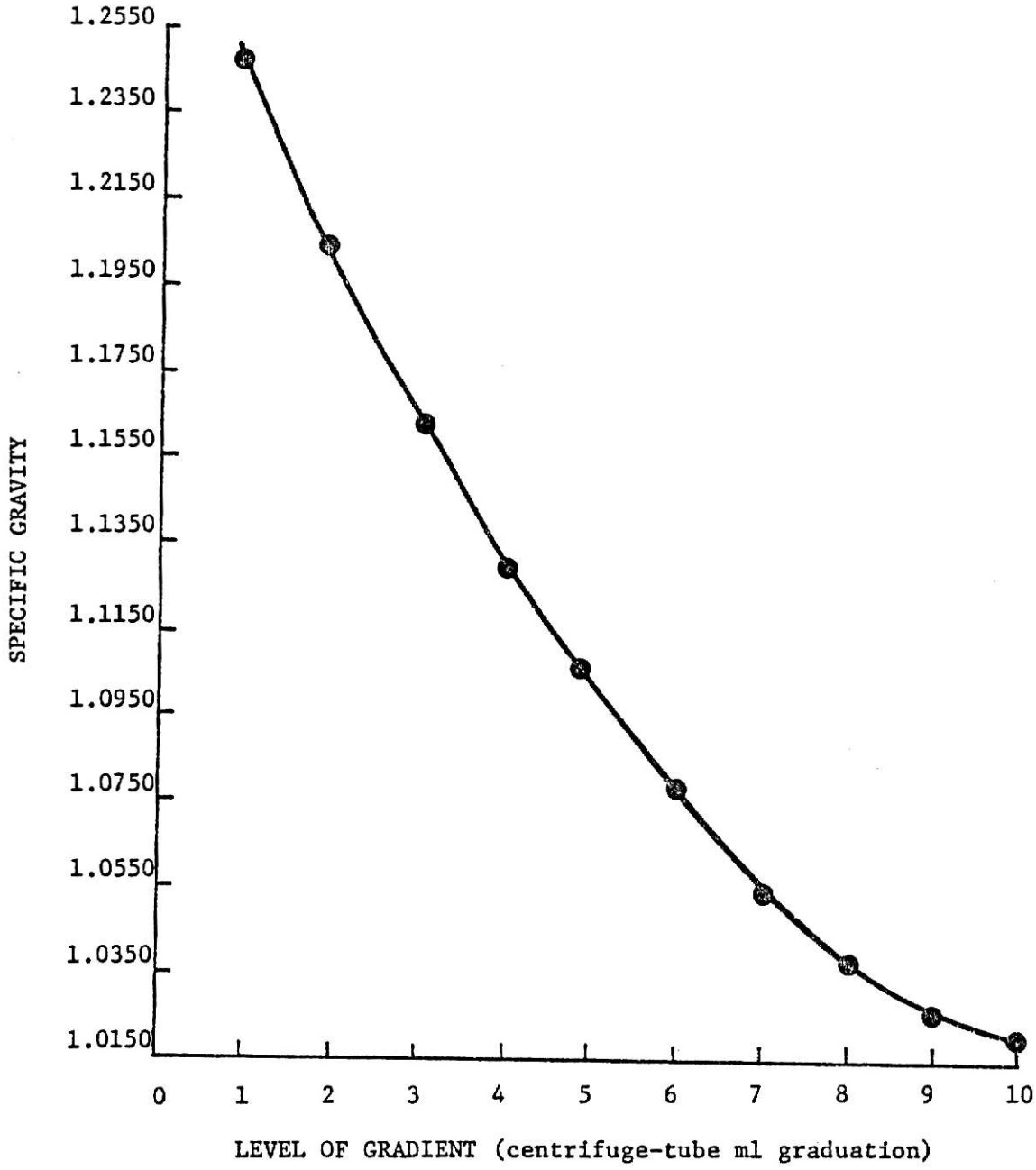
Worker/s	Washing agent	Flotation solution	Manner of egg recovery	Other treatments	Recovery efficiency[1]
World Health Organization (1967)	NaOCl	NaCl	supernatant from flotation centrifuged, eggs recovered in sediment	sample screened prior to flotation	ND
Romanenko (1968) (from Chefranova, 1979)	water	saturated $NaNO_3$	eggs recovered on coverslip	sample mixed with water in Gudzabidze's mixer	71.6%
Isaev et al. (1969)	-	-	aliquots removed from suspension	air blown through sample in water	"100% or nearly 100% positive results"
Borg and Woodruff (1973)	water	$ZnSO_4$ (sp gr 1.18)	supernatant from flotation passed through millipore filter	-	ND
Dubin et al. (1975)	Tween 60	saturated $NaNO_3$	eggs recovered on slide	sample filtered prior to flotation	ND
Sewell and Urquhart (1976)	1% Tween 80	-	supernatant from centrifugation passed through 2 millipore filters	-	ND

APPENDIX 1. (Continued)

Worker/s	Washing agent	Flotation solution	Manner of egg recovery	Other treatments	Recovery efficiency[1]
Theis et al. (1978)	water	saturated $ZnSO_4$ (sp gr 1.18)	aliquots removed from surface	sample screened prior to flotation	ND
Oganov et al. (unpubl.)(from Chefranova, 1979)	5% NaOH	saturated $NaNO_3$	supernatant filtered	sample mixed with NaOH in Gudzabidze's mixer	ND
Dada and Lindquist (1979)	0.1N NaOH	$ZnSO_4$ (sp gr 1.20)	eggs recovered on 5 coverslips (5 flotations)	inner wall of centrifuge tube scraped with metal wire prior to each centrifugation after the first	61.95-67.52% [Toxocara]
Quinn et al. (1980)	0.0025% Tween 80	saturated $MgSO_4$+5% KI	eggs recovered on 4 coverslips (4 flotations)	sample screened prior to flotation	ND

[1]Identity of eggs used when given is bracketed.

[2]ND = no data given.



APPENDIX 2. Specific gravity slope of sucrose density gradient 48 hr after preparation. Values plotted are means of measurements at different levels of four gradients.

APPENDIX 3. Comparison of specific gravity determinations from the present study with previous determinations.

Species	Specific gravity	
	Previous studies	Present study
Ancylostoma duodenale	1.030-1.340 (Mhaskar, 1923)	ND[1]
Nematodirus spathiger	1.050-1.075 (Marquardt, 1961)	ND
Necator americanus	1.055 (Sawitz et al., 1939)	ND
Ancylostoma caninum	1.055 (Sawitz, 1942)	1.0559
Toxascaris leonina	1.080-1.120 (Kolomysov, 1978)	1.0559
Toxocara canis	1.070-1.120 (Kolomysov, 1978)	1.0900
Parascaris equorum	NPR[2]	1.0969
Toxocara cati	1.075-1.120 (Kolomysov, 1978)	1.1005[3]
Ascaris lumbricoides	1.110-1.130 (Sawitz, 1942)	ND
Enterobius vermicularis	1.115 (Sawitz, 1942)	ND
Ascaris suum	1.1036 (Magat et al., 1972) 1.130-1.180 (Kolomysov, 1978)	1.1299
Trichuris suis	NPR	1.1299
Trichuris trichiura	1.150-1.160 (Sawitz, 1942)	ND
Trichuris vulpis	1.150-1.160 (Sawitz, 1942) 1.150-1.185 (Kolomysov, 1978)	1.1453

APPENDIX 3. (Continued)

Species	Specific gravity	
	Previous studies	Present study
Taenia sp.	NPR	1.2251
Physaloptera sp.	NPR	1.2376

[1]ND = Not done.

[2]NPR = No previous reports.

[3]Embryonated.

APPENDIX 4. Details of materials and methods.

Section I

A. Source of Eggs and Method of Concentration

Fecal samples were collected from the Animal Shelter at Sunset Zoo in Manhattan, Kansas. These samples were examined by sodium chloride or zinc sulfate flotation and those that were strongly positive for Toxocara canis and Trichuris vulpis eggs were processed in the following manner: feces was mixed thoroughly with tap water and when necessary, blended in a laboratory blender for 20 sec. The resulting fecal suspension was passed through a 60-mesh screen into a 1,000-ml graduated cylinder. The material retained on the screen was then washed with more tap water until two graduated cylinders were filled. These were then allowed to stand overnight at 4 C. The following day, the supernatant was drawn off using an aspirator and water added to the sediment until the original volume was reached. The cylinders were then stored overnight again at 4 C. This process was repeated until the supernatant was relatively clear. This final supernatant was then aspirated off and aliquots of the sediment transferred into ground-top centrifuge tubes. Each tube was filled to the brim with $ZnSO_4$ solution (sp gr 1.20), topped with an 18x18-mm #1 coverslip, and centrifuged at 158 g for 5 min. Following centrifugation, the coverslips were removed and their undersides washed into a low, wide-mouthed, screw-cap glass jar with 0.5% formalin. This egg suspension was stored at 4 C until further use.

Ascaris suum-positive swine feces was obtained from barnyard-raised pigs and was processed in a similar manner.

B. Determination of Concentration of Egg Suspension for Seeding of Soil Samples

The jar containing the egg suspension was uncapped and secured on a flask clip on a shaker head attached to a Vortex-Genie mixer. The mixer was turned on at low speed, and while the jar was being gently shaken, a Biopette automatic pipette (Carworth, New City, N. Y.) was lowered to the suspension and a 0.05-ml sample was drawn. This was put on a glass slide, a coverslip applied, and the eggs counted. Four such samples were taken and examined and the average count/ 0.05 ml was calculated. From this was extrapolated the quantity of the suspension that would contain approximately 20,000 eggs to use to seed 100 Gm of soil. A sample calculation follows: counts of Toxocara canis eggs in four 0.05-ml aliquots of T. canis egg suspension were: 168, 184, 177 and 185. Average count was 179. Thus, in 1 ml of this suspension there were 3,580 eggs (179 X 20) and 5.6 ml will contain approximately 20,000 eggs (20,000/ 3,580).

C. Source of Soil and Method of Seeding

Clay soil was obtained using a garden trowel from the grounds of the Veterinary Medical Teaching Building, Kansas State University. This was sterilized at 82 C for 30 min to destroy any possible nematode egg contamination.

One hundred grams of sterilized clay soil was seeded with approximately 20,000 eggs as follows: 50 Gm of soil was placed in a laboratory blender. Half of the quantity of egg suspension previously calculated to contain 20,000 eggs was pipetted off from the container while the latter was being shaken gently on a Vortex-Genie mixer and added to the soil in the blender. The soil was then blended for 1 min. Following this, it was mixed thoroughly with a rubber spatula, aggregates clinging to the sides and cover of the blender being

scraped into the rest of the sample at the bottom. This process of alternate blending and manual mixing was repeated several times and then the other half of the calculated quantity of egg suspension was added. Alternate blending and manual mixing was once again carried out. The soil was then transferred into a Dispo cup and stored at 4 C until further use.

D. Preparation of Solutions

Decinormal sodium hydroxide was prepared by dissolving 4 Gm of NaOH in 1 ℓ of distilled water.

Zinc sulfate (sp gr 1.20) was prepared by dissolving 385 Gm of $ZnSO_4$ in 1 ℓ of distilled water. Specific gravity was checked using a hydrometer.

Section II

A. Source of Eggs and Method of Concentration

Fecal samples were collected from the following facilities: experimental animal runs and horse stalls, KSU Veterinary Hospital; Animal Shelter, Sunset Zoo; Howser Stables; and a barnyard piggery, all in Manhattan, Kansas. These samples were qualitatively screened by zinc sulfate flotation and the ones that were strongly positive for the appropriate helminth eggs were processed into fecal or egg concentrates.

A fecal concentrate was prepared by screening the sample and then washing it in tap water by repeated sedimentation and decantation as in Section I. The final sediment was suspended in distilled water and stored at 4 C until further use. Immediately before use, the supernatant water was drawn down to just above the level of the sediment so that when the latter was resuspended, a thick suspension was formed. This final material constituted a fecal concentrate.

Egg concentrates were prepared from fecal concentrates as follows: aliquots of fecal concentrate were transferred into 12-ml centrifuge tubes which were then centrifuged at 158 g for 5 min. The supernatant was decanted from each tube and the sediment resuspended in $ZnSO_4$ soltuion (sp gr 1.20). The tube was then trimmed with more $ZnSO_4$, an 18x18-mm #1 coverslip was applied, and centrifugation was carried out at 158 g for 5 min. Following this, the coverslips were removed and their undersides washed with distilled water into two 12-ml centrifuge tubes. These tubes were then centrifuged at 158 g for 5 min. The supernatant was decanted, the sediment resuspended in distilled water, and the tubes recentrifuged. This washing process was performed four or five times. The final sediment was resuspended in 8-10 ml of distilled water and stored in a test tube at 4 C until further use.

The Toxocara cati-positive fecal sample was submitted to the laboratory by a private practitioner and was processed in a similar manner. These eggs were embryonated when the sample was received.

B. Preparation of Sucrose Solutions

The sucrose solutions used to prepare gradients were prepared from sucrose as follows: the appropriate quantity of sucrose to make 100 ml of each solution was weighed on an analytical balance. This was 3.03 Gm for the 3% solution, 13.66 Gm for the 13% solution, 26.38 Gm for the 24% solution, 40.29 Gm for the 35% solution, and 67.60 Gm for the 54% solution. These quantities were each dissolved in enough distilled water to make 100 ml of final solution in a 100-ml volumetric flask. The refractive index of each solution was checked prior to its use using a Bausch and Lomb Abbe-3L refractometer. The solutions were stored in screw-cap glass bottles at 4 C while not in use.

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EVALUATION OF A MODIFICATION OF THE DADA TECHNIQUE FOR THE RECOVERY OF HELMINTH EGGS FROM SOIL, AND DETERMINATION OF THE SPECIFIC GRAVITY OF CERTAIN HELMINTH EGGS USING SUCROSE DENSITY GRADIENT CENTRIFUGATION

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ABSTRACT

A modification of the Dada technique for the recovery of helminth eggs from soil was devised and evaluated. The modified technique consisted of washing of the sample with decinormal sodium hydroxide or tap water and centrifugal flotation in zinc sulfate solution (sp gr 1.20), followed by centrifugal flotation in the same solution of the supernatant and sediment separately. The supernatant and sediment from the third centrifugation were similarly processed for a total of five coverslip recoveries.

The modified technique was evaluated using sterilized clay soil artificially seeded with 200 nematode eggs/ Gm. It was most efficient in recovering seeded Trichuris vulpis eggs, retrieving 63.80% using NaOH and 72.40% using tap water. It showed moderate recovery efficiency for Toxocara canis eggs, recovering 54.95 and 55.83% using NaOH and tap water, respectively. It showed poor recovery efficiency for seeded Ascaris suum eggs, recovering only 27.90 and 10.60% using NaOH and tap water, respectively. Recovery efficiency rates of the technique using NaOH and tap water were statistically different ($P<0.05$) in the case of T. vulpis and A. suum.

At least 95% of all eggs recovered in each trial were retrieved on the first three or four coverslips, depending on the species involved and the washing agent used. Three coverslips recovered at least 85% of all recoverable eggs in all trials.

The specific gravities of ten species of helminth eggs were determined using sucrose density gradient centrifugation. Fecal or egg concentrate was layered over a 3-54% sucrose density gradient. This was then centrifuged at 800 g for 20 min, allowing 5 min for acceleration and 5 for deceleration. Bands formed were identified and measured. Refractive index was measured at

the middle of narrow bands or at the level at which the concentration of eggs was highest, in the case of wide bands. This level was determined by drawing a 0.05-ml sample at every 0.1-ml graduation on the centrifuge tube within a span of the gradient that included the band, and counting the eggs in each sample. When no band was formed, the eggs were first located by sampling from every 0.5-ml graduation on the centrifuge tube and examining the samples microscopically for eggs. Subsequently, using a second gradient, a 0.05-ml sample was drawn at every 0.1-ml graduation on the centrifuge tube within a span of the gradient that included all levels found positive for eggs in the preliminary experiment. The eggs in each sample were counted and refractive index was measured at the level that yielded the highest egg count. The specific gravity corresponding to this refractive index was taken as the specific gravity of the respective helminth eggs.

The specific gravities of the following helminth eggs as measured on three or four gradients were: Toxascaris leonina and Ancylostoma caninum, 1.0559; Toxocara canis, 1.0900; Parascaris equorum, 1.0969; Toxocara cati (embryonated), 1.1005; Ascaris suum and Trichuris suis, 1.1299; Trichuris vulpis, 1.1453; Taenia sp, 1.2251; and Physaloptera sp, 1.2376. These determinations agreed with or approximated those of previous workers. The specific gravities of P. equorum, T. suis, Taenia sp and Physaloptera sp eggs are reported for the first time.