Improved methods for recovering eggs of *Toxocara canis* from soil

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Abstract

The ingestion of soil in parks and public places containing eggs of *Toxocara* may constitute a significant health risk, particularly to children. To determine the most efficient method for extracting eggs from experimentally contaminated soil, two consecutive studies were undertaken. Four techniques, including washing, sieving, vacuum, and the one recommended by the World Health Organization, were evaluated. Recovery rates of over 85% were recorded with both washing and sieving methods. Using the washing technique, all combinations of the four pre-treatment solutions, distilled water, acetoacetic solution pH 5, 0.1 N sodium hydroxide and 1% Tween 20, and seven flotation fluids with different specific gravities (S.G.) ranging from 1.20 to 1.35 were assayed. The association of distilled water and saccharose solution with an S.G. of 1.27 showed the best results, with a recovery rate of 99.91%.

Introduction

Visceral larva migrans (VLM) is a zoonotic disease caused by migration of larval nematodes through extra-intestinal tissues of unnatural or unsuitable hosts (Shantz & Glickman, 1978). Although several nematodes have been associated with VLM, the roundworm of dogs, *Toxocara canis*, has been most frequently related with this infection. The roundworm of cats, *Toxocara cati*, may also be of potential significance in VLM (Childs, 1985). Additionally, experimental infections with eggs of *Toxascaris leonina* produce a similar VLM picture (Quinn *et al*., 1980). The risk of infection in man is greatest in young children between 1 and 4 years of age. Complete reviews of this clinical syndrome have already been made (World Health Organization, 1967; Ehrhard & Kerbaum, 1979; Magnaval *et al*., 1994; Prieto Novoa *et al*., 1995). *Toxocara canis* eggs require 2–5 weeks after excretion to develop to infectivity (Shantz & Glickman, 1983). Therefore, human infection usually occurs by accidental ingestion of embryonated eggs with soil or carried on contaminated hands or objects rather than through direct contact with an infected animal (Shantz & Glickman, 1978; Barriga, 1988; Nunes *et al*., 1994). Public parks, particularly those heavily fouled by dogs and cats, are an important source of infection (Borg & Woodruff, 1973; Gillespie *et al*., 1991).

The methods usually employed for faecal examination cannot be used with soil because of the strong attachment of ascarid eggs to soil particles (World Health Organization, 1967), and several chemical or physical procedures have been proposed to separate them (Dada, 1979; Dada & Lindquist, 1979; Laborde *et al*., 1980; Quinn *et al*., 1980; Kazacos, 1983; O'Lorcain, 1994; Ajala & Asaolu, 1995). Numerous studies have been made using different techniques, sample pre-treatment solutions and flotation fluids (World Health Organization, 1967; Dada, 1979; Quinn *et al*., 1980; Laborde *et al*., 1980; Kazacos, 1983; Shimizu, 1993; O'Lorcain, 1994; Ajala & Asaolu, 1995). The aims of the present study are to evaluate the efficiency of four different methods for recovering ascarid eggs from soil and also increase efficiency by employing pre-treatment of samples with several electrolyte solutions and seven flotation fluids.

Materials and methods

Preparation of soil samples

Sandy soil samples collected from a public park in Espinardo (Murcia, Spain) were placed in an oven at 200°C for 60 min to dry until they maintained constant weight, to destroy any eggs before artificial seeding. After

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drying, samples were passed through a sieve of 3.4 mm^2 pore size to remove stones and large debris.

Seeding of soil samples with Toxocara canis *eggs*

Eggs of *T. canis* were obtained from canine faeces using the formol-ether concentration technique (Allen & Ridley, 1970). Faecal sediments were suspended in normal saline solution to create a stock of eggs. Ten petri dishes each with 100 g of sterilized sandy soil were prepared to compare the efficiency of different laboratory procedures for egg recovery from soil. Each one was seeded to a final concentration of 543 eggs g^{-1} of soil.

For testing pre-treatment and flotation solutions, 400 g of sterilized sandy soil was distributed in four petri dishes (100 g per dish); each of which was seeded with 15 ml of the faecal suspension containing 280,400 eggs (2804 eggs g^{-1} of soil). Both kinds of samples were processed separately as follows.

The experimentally contaminated samples were mixed using an electric laboratory blender for 1 min. After blending, soil particles were allowed to settle and the samples transferred to a container. A rubber spatula was used to clean the inside walls of the blending machine and the residual soil was added to the container.

Laboratory procedures

Four methods were compared and ten replicates of each one were performed.

Washing technique (Dada, 1979)

Ten aliquots of 1 g of contamined soil were weighed and transferred into 12 ml centrifuge tubes, and 9 ml of distilled water added to each tube. Samples were shaken for 1 min and centrifuged at 1500 *g* for 5 min. After discarding the supernatant, saccharose (S.G. 1.27) was added as a flotation solution to complete a volume of 10 ml. The mixture was shaken and used to fill five McMaster chambers, which were examined microscopically for the presence of *T. canis* eggs.

Sieving technique (Laborde *et al*., 1980)

Ten samples of 50 g of soil were first washed with running tap water using a set of three sieves with mesh widths of 300 μm, 120 μm and 63 μm. The residue in the last sieve was flushed into a 250 ml container, the mixture allowed to settle and the liquid then decanted following a 15 min sedimentation period. The pellet was collected into 50 ml centrifuge tubes and shaken with the saccharose (S.G. 1.27) flotation solution, prior to examination for *T. canis* eggs.

Vacuum technique

This was performed using a modified version of Quinn *et al*. (1980) and O'Lorcain (1994). To each of ten 15 g samples analysed, 25 ml of distilled water was added. Soil samples were mixed for 3 min and passed through a 1 mm^2 nylon sieve into a Büchner funnel using a suction pump attached to a flask. The filtrate was transferred to 50 ml centrifuge tubes and centrifuged at 1500 *g* for 5 min. The supernatant was discarded, the pellet resuspended in the flotation solution (saccharose, S.G. 1.27) and the presence of eggs determined using the McMaster chambers as previously described.

World Health Organization (WHO) technique

Ten samples, each comprising 20 g of contaminated soil, were treated with 200 ml of 30% bleach in tap water and allowed to stand for 30 min with agitation. Water (375 ml) was added to the mixture which, after shaking, was allowed to sediment for 10–15 s to settle the macroscopic particles. The liquid was decanted into 50 ml centrifuge tubes and the sediment discarded. After centrifuging at 1500 *g* for 5 min, the pellet was analysed in the same saccharose flotation solution.

Once the most efficient laboratory procedure had been selected, several pre-treatment solutions and flotation fluids were evaluated. Ten replicates of each possible combination were performed.

Pre-treatment solutions

Soil samples were mixed with different solutions in order to facilitate separation of eggs. Four solutions were tested: distilled water, 0.1 N sodium hydroxide, and 1% (v/v) Tween 20 in distilled water and 0.2 M acetoacetic solution pH 5.

Flotation fluids

The solutions under test were made up and the specific gravities determined at 21°C. The following aqueous solutions were used: saccharose (S.G. 1.20), saccharose (S.G. 1.27), saturated zinc sulphate (S.G. 1.20), saturated sodium chloride (S.G. 1.20), magnesium sulphate (S.G. 1.28), magnesium sulphate plus 5% potassium iodide (S.G. 1.32) and sodium nitrate (S.G. 1.35).

Data analysis

Egg counts of *T. canis* were transformed to the natural logarithm (counts + 1) for statistical evaluation (Zar, 1984). Analysis of variance was performed first within each of the four methods. Within the most efficient method, the variance was analysed between the pre-treatment solutions and the flotation fluids (multiple ANOVA). Since variances were not assumed to be equal, a Brown-Forsythe test was used (Brown & Forsythe, 1974). A Student-Newman-Keuls multiple range was performed as a post-hoc test and the analyses performed using a BMDP package (BMDP Statistical Software, Inc., 1985).

Results

Laboratory procedures involving washing and sieving, respectively were more efficient than the others ($P < 0.01$), with more than twice as many eggs being recovered (table 1). Up to 99.91% of eggs were recovered using the washing method compared with 89.43% using the sieving technique, although the differences between these two methods were not significant.

Pre-treatment with 0.1 N sodium hydroxide and 1% Tween 20 always showed very low recovery rates (table 2)

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Table 1. Recovery of *Toxocara canis* eggs from contaminated soil: a comparison of laboratory procedures.

Methods	Recovered eggs per gram of soil		
	x̄	SE	Percentage
Washing	542.52[a]	41.14	99.91
Sieving	485.62	36.12	89.43
Vacuum	211.03	22.57	38.86
WHO	207.99	16.03	38.30

x̄, Mean number of eggs; SE, standard error; %, mean percentage.
[a] Refers to ten soil samples.
Experimental conditions: eggs g^{-1} of soil = 543; flotation fluid: saccharose solution (S.G. 1.27).

although the rates improved with distilled water and acetoacetic solution. Variance analysis showed statistically significant differences ($P < 0.01$) in the recovery of *T. canis* eggs amongst the pre-treatment solutions, although a comparison between distilled water and acetoacetic solution showed less significance ($P < 0.05$). Statistically significant differences ($P < 0.01$) were shown in the recovery of *T. canis* eggs amongst all the flotation fluids.

Statistical analysis indicated the presence of significant interactions between both factors, i.e. pre-treatment and flotation fluids and in particular, saturated sodium nitrate solution (S.G. 1.35) showed significant differences ($P < 0.01$) with the combination of distilled water and saccharose solution (S.G. 1.27), with a maximum rate of recovery of *T. canis* eggs (99.98%).

Discussion

The recovery of helminth eggs such as those of *T. canis* from soil allows an evaluation of human risk of being affected by VLM. For this epidemiological purpose, simple and efficient methods for the isolation of eggs from soil samples are required.

A comparison of four laboratory procedures revealed that there were statistically significant differences among them. In this study, the WHO technique (1967) showed the poorest results, with an egg recovery rate of 38.30%. Laborde *et al*. (1980) used a method derived from that proposed by WHO, but they avoided the addition of bleach. Ajala & Asaolu (1995) reduced the initial 30% dilution of bleach proposed by WHO to 20%. Gaspard & Schwartzbrod (1993) showed that high chlorometric degrees of sodium hypochlorite solution (25 to 15) could destroy eggs by oxidation. Also, excessive exposure to this hypochlorite solution may affect the external coating of eggs and thus prevent them from floating correctly. We also found some technical difficulties with the vacuum technique as part of the sample remained attached to the nylon sieve, the Büchner funnel or the container. Also, many particles, which were distributed throughout the saccharose solution, were physical obstacles, which impeded the flotation of helminth eggs. All these factors might influence the low rates observed with this technique.

However, recovery rates were high if sieving or washing techniques were employed, with recovery rates of 89.43% and 99.91%, respectively but no statistical differences were demonstrated between these two methods. The recovery rate of the washing technique was higher than that obtained by Dada (1979) for clay soil (66.19%) and this could be due to differences in the specific gravity of the floating solutions and the soil characteristics. The best results in his study were obtained when zinc sulphate solution (S.G. 1.20) was employed. In previous surveys (Nunes *et al*., 1994; Ajala & Asaolu, 1995) soil texture appears to be an important variable. It was suggested that contaminated samples from sandy soils were more homogeneous, and the larger-sized soil particles held the eggs more loosely than in the case of clay soils. Hence, better recovery ratios were always observed in sandy soil. Dada (1979) used clay soil whereas in this study samples were obtained from a carefully sieved sandy one.

The present results differed from those of Kazacos

Table 2. Recovery of *Toxocara canis* eggs from contaminated soil: a comparison of pre-treatment and flotation solutions.

Flotation solution (S.G.)	Pre-treatment solution											
	Distilled water			0.1 N Sodium hydroxide			1% Tween 20			Acetoacetic pH 5		
	x̄	SE	%	x̄	SE	%	x̄	SE	%	x̄	SE	%
Saccharose (1.20)	90[a]	15.94	3.21	60	14.88	2.14	60	13.26	2.14	110	18.81	3.92
$ZnSO_4$ (1.20)	230	31.19	8.20	176.67	29.13	6.30	163.33	28.76	5.82	383.33	43.19	13.67
NaCl (1.20)	76.67	18.57	2.73	186.67	26.36	6.66	86.67	17.33	3.09	300	40.12	10.70
Saccharose (1.27)	2803.33	141.56	99.98	990	76.25	35.31	816.67	50.90	29.12	1096.67	75.77	39.11
$MgSO_4$ (1.28)	650	49.77	23.18	633.33	51.95	22.59	320	31.17	11.41	1406.67	63.92	50.17
$MgSO_4$·KI (1.32)	530	35.85	18.90	380	33.35	13.55	196.67	22.18	7.01	703.33	45.00	25.08
$NaNO_3$ (1.35)	1443.33	73.86	51.47	1113.33	76.10	39.70	250	27.04	8.91	2323.33	93.73	82.86

x̄, Mean number of eggs per gram of soil; SE, standard error; %, mean percentage.
[a] Refers to ten soil samples; SG, specific gravity.
Experimental conditions: eggs g^{-1} of soil = 2804.

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(1983), who proposed the use of large amounts of soil for improving egg recovery rates. This should be of critical importance when samples are naturally contaminated, since they are likely to contain a low concentration of eggs. However, when artificially seeded soil was used in the present study, sample size did not appear to influence the results.

Washing of the sediment before flotation is advantageous in removing fine soil particles, but the present results revealed that neither Tween 20 or 0.1 N sodium hydroxide solution increased the rate of *Toxocara* egg recovery. Our results agree with the findings of Gaspard & Schwartzbrod (1993), who obtained very low recovery percentages using similar pre-treatment solutions but disagree with those of Dubin *et al*. (1975), Quinn *et al*. (1980) and Kazacos (1983) who showed that the anionic detergents Tween 60, 80 and 40, respectively, improved egg recovery. On the other hand, the present results agree with those of Dada & Lindquist (1979) who found that 0.1 N sodium hydroxide was unnecessary as tap water produced better results. Pre-treatment with acetoacetic solution produced the best results in the present study when combined with the majority of the flotation fluids. Only when saccharose solution (S.G. 1.27) was used, did pre-treatment with distilled water reveal a better egg recovery rate and this selected combination showed statistically significant differences when compared with other tested solutions.

According to Kazacos (1983) and Ajala & Asaolu (1995), the recovery of eggs from samples floated with sodium nitrate solution (S.G. 1.35) was high. However, our results differ from those of Kazacos (1983) in saccharose solution efficiency for *T. canis* egg recovery. The best results were obtained using this solution, and statistical analysis revealed significant differences when compared with other flotation solutions. This may be explained by the greater specific gravity of the saccharose solution in this study (S.G. 1.27) when compared with that used by Kazacos (1983) (S.G. 1.24).

The present results support previous findings that salt solutions with higher specific gravities are more effective in floating helminth eggs from soil (Quinn *et al*., 1980; Ajala & Asaolu, 1995). However, magnesium sulphate plus 5% potassium iodide (S.G. 1.32) revealed lower egg recoveries, suggesting that physico-chemical properties of solutions such as viscosity may be important (Quinn *et al*., 1980; Kazacos, 1983; Nunes *et al*., 1994). Similar results were obtained by Dada & Lindquist (1979) as the lowest egg recovery was recorded using a solution with a high specific gravity (mercuric iodide, S.G. 1.63). Furthermore, differences in the rate of egg recovery in solutions with similar specific gravities of 1.20, such as saccharose, saturated zinc sulphate and saturated sodium chloride could be attributed to unspecified physico-chemical properties. Dada (1979) for example, obtained better results using zinc sulphate than sodium dichromate, both with a specific gravity of 1.20. On the other hand, Quinn *et al*. (1980) showed that zinc sulphate (S.G. 1.20), a solution widely used for the recovery of eggs from faecal samples, performed extremely poorly as did another frequently recommended solution, sodium chloride (S.G. 1.20).

Values of egg recovery of up to 100% are most unusual, although recovery percentages of over 90% were observed in previous assays. Dunsmore *et al*. (1984) obtained values of 91.4%, and Gaspard & Schwartzbrod (1993) recorded values of 83 to 100% when loamy or sandy samples, respectively, were used. In similar standardization studies, using samples from municipal compost, Steer *et al*. (1974) cited recovery percentages from 93.8 to 100%. Wong & Bundy (1990) also found recovery values of up to 100% but pointed out that overestimation is intrinsic to the standardization methods, and these records should therefore be regarded as of relative rather than absolute significance.

Important variables, such as soil type, climatic conditions (temperature, rainfall, sunlight, etc.) or the amount of herbage, are unlikely to influence the efficiency of the recovery procedures compared in the present study, as the infected soil was collected on a determined date from one habitat. The present study also showed that the washing technique derived from the method of Dada (1979), using distilled water as pre-treatment and saccharose solution (S.G. 1.27) as the flotation fluid showed an adequate egg recovery efficiency and this was sufficiently simple to be used under field conditions.

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