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ISOLATION OF *EIMERIA* OOCYSTS FROM SOIL SAMPLES: A SIMPLE METHOD DESCRIBED IN DETAIL

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ABSTRACT. Molecular methods are currently available to detect parasitic DNA in soil, but do not replace the need for direct parasitological methods that can identify the extent of contamination. This report describes an inexpensive and fast flotation method for retrieving *Eimeria* oocysts from soil that requires few tools. Soil samples were experimentally contaminated with 50,000 *E. bovis* oocysts and compared with soil-free controls. A separate experiment tested the effect of mechanical stress by shaking the soil and oocyst mix 0, 1, 5, and 10 times before attempting to retrieve the oocysts. The percentage of oocysts retrieved using the flotation method was 22%, which was similar to the results obtained with previously described, more labour-intensive methods. The presence of soil reduced the percentage of oocysts that could be retrieved by 23%. A single shake of the oocysts and soil mixture was sufficient to significantly reduce the recoverable proportion of oocysts. The results indicated that the developed simple method can be applied to recover oocysts, and that gentle handling of soil samples prior to oocyst isolation is important.

Introduction

Many parasites relevant to humans, wildlife, and domesticated animals are shed in faeces and spend a large proportion of their existence in the environment. During the biological breakdown of the faecal pat and exposure to rain and insects, the parasites can become mixed into the soil. From the soil, the environmentally resistant stages of the parasite (eggs, cysts, and oocysts) can enter hosts that ingest the soil, drink from water sources that are in contact with the soil, or eat vegetation growing in the soil. Although the soil phase is crucial for many animal parasites, the presence and persistence of these in soil, and particularly for bovine species of *Eimiera*, has not been studied in great detail. Without this knowledge, the ability to evaluate environmental risks, and thus plan prevention, is limited.

A major limitation to the progress of research in parasite ecology in soil has been the lack of sensitive quantitative methods. Molecular methods exist to detect DNA of parasites in the soil (Durant *et al*., 2011; Lélu *et al*., 2011; Koken *et al*., 2013; Tavalla *et al*., 2013), but are expensive, laborious, or offer only an indirect method of quantifying the presence of parasites. Such methods are not yet able to evaluate the ability of a parasite egg or oocyst to develop to an infective stage. Molecular methods thus do not yet offer a practical alternative to isolation of the parasite directly from the soil. The development of methods for the collection of data on environmental contamination with parasites is therefore needed (Lélu *et al*., 2011). Oocysts can be difficult to detect and are more fragile than the larger and more robust parasite eggs. Many existing flotation methods are complicated and time consuming, require special tools and reagents, and have had only moderate success in quantifying oocysts in soil (Jenkins *et al*., 2002; Kato *et al*., 2002; Ramirez, Sreevatsan, 2006; Lélu *et al*., 2011). A search for a specific protocol for isolating *Eimeria* oocysts from soil in the available literature was unsuccessful. The isolation of *Eimeria bovis* oocysts from soil was first attempted using a protocol for retrieving nematode eggs from soil (Mejer *et al*., 2000) but there were substantial losses in the recovery during sieving and centrifugation steps were observed. As a consequence a method was devised with satisfactory analytical sensitivity which would also be simple, inexpensive, fast, and require no special equipment besides a microscope. Previously the method was applied to demonstrate that species of *Eimeria* do survive natural climate conditions in the soil where bovine faecal pats

have previously been deposited (Lassen *et al.,* 2014). Other studies have shown that feeding or providing water for cattle from sources in contact with possibly contaminated soil increase the risks of acquiring *Eimeria* infections (Svensson, 1997; Rehman *et al.*, 2012). In this article, a new recovery method is described in detail and the effect of gentleness of handling on the recovery of *E. bovis* is assessed.

Methods

The first experiment (efficacy of oocyst isolation by gentle flotation from soil) and the second experiment (the effect of non-gentle handling) were carried out by two different persons.

Soil material. The soil was collected in Estonia (58°19′18.05″N, 26°49′20.79″E). The agricultural area had not been in use for three years and had no record of grazing by livestock. Larger roots and rocks were removed and the remaining soil was dried at 70°C for 5 days. The dried soil was analysed and estimated to be sandy soil (80.62% sand, 12.31% silt, 7.07% clay, and 0.96% organic carbon).

Oocysts. The *Eimeria bovis* oocysts used in the experiment were a German isolate (LE-10-E, Institute of Parasitology, University of Leipzig, Germany). The oocysts were isolated in April 2010 from calf faeces, as previously described (Lassen *et al*., 2013). A haemocytometer (Bright-Line, Hausser Scientific, USA) was used to determine the concentration of oocysts in solutions, and dilutions were made using tap water.

Gentle flotation from soil. Soil was added to 50 ml syringes (Nipro Syringe Catheter Tip 50 ml, NIPRO Europe, Poland) in 10.04 g (± 0.09 STDV) aliquots before spiking each with 50,000 12-month-old *E. bovis* oocysts suspended in 1 ml of tap water. The oocysts were left for 30 min at room temperature before recovering them as described in Figure 1. The volume of the sugar-salt flotation liquid (specific gravity = 1.24) collected from the syringe (step 11, Figure 1) was recorded as the volume of dilution (Vd) before mixing and adding to a flotation chamber constructed with microscope slides as designed by Henriksen and Korsholm (1984). The chambers are illustrated in Figure 2. After 5 min, sufficient time to allow oocysts to float, three vertical rows were examined at ×200 magnification (Vr = 0.0648 ml) and the oocysts were counted. The calculation of different volumes is described in Figure 2 for both the constructed chamber used and McMaster chambers. The total oocyst count was calculated as Vd × (N / Vr), where N is the number of oocysts, Vd is the volume of sugar-salt flotation liquid collected from the syringe, and Vr is the examined volume of the reading chamber. The experiment was carried out with twenty repetitions.

The calculations used for determining oocyst counts from wet soil have been previously described by Lassen *et al.* (2013). Four negative controls were included in the experiment.

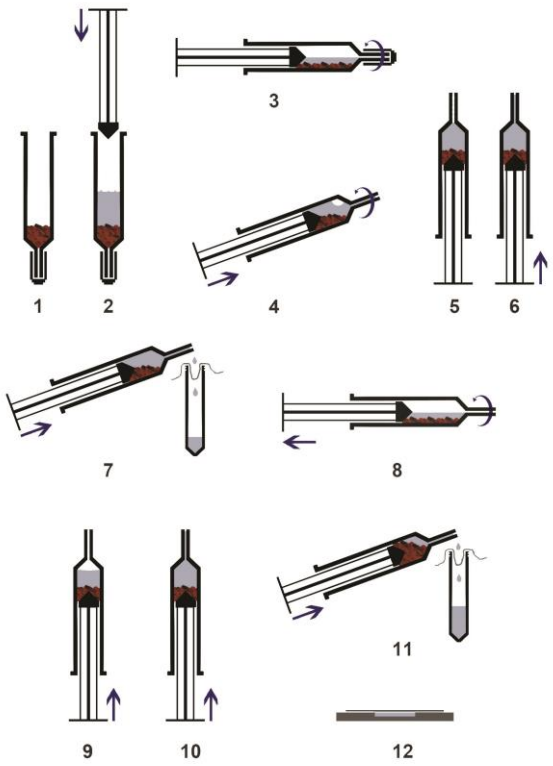

Figure 1. Gentle flotation method for recovering oocysts from soil: 1) First, position a 50 ml syringe vertically and securely with the tip cap on, and then weigh 10 g of soil in the syringe. 2) Add 20 ml of sugar-salt flotation liquid into the barrel and insert the plunger. 3) Rotate the syringe so that the tip now points upwards. Remove the cap to release pressure, and push the plunger half-way in (use another needle to press down any soil stuck in the needle, or tap gently by flicking with a finger against the tip). Replace the cap, and then position the syringe horizontally and rotate slowly at 5 minute intervals for 30 min. 4) Move the syringe carefully to a 45 degree angle. Remove the cap and press the liquid to the tip. An air bubble should be present in the upper corner of the syringe. Rotate the syringe at 45 degrees using the air bubble to push oocysts around along the narrowing edge of the syringe. 5) Raise the syringe vertically allowing the bubble and possible oocysts to be moved up the tip by the bubble. 6) Press liquid to the tip and wait 15 minutes for oocysts to flotate. 7) Press approximately 3 ml into a 14 ml tube through a piece of gauze. 8) Retract the plunger half-way and rotate the syringe slowly to dispense the soil. 9) Raise the syringe tip upwards and push the plunger back such that the liquid is just below the entrance to the tip and wait 15 minutes. 10) Press the plunger so that the liquid stops at the tip and wait 5 minutes. 11) Press 3–5 ml of the remaining liquid into the same 14 ml tube through a piece of gauze, as in step 7 (avoid pressing soil through). 12) Note the volume of liquid in the 14 ml tube. Mix the liquid with a plastic pipette and transfer to a reading chamber. Wait 5 minutes before reading to allow the oocysts to flotate.

The loss of detection of oocysts that could be attributed to the presence of soil was evaluated by comparison with soil-free controls: 10 ml of flotation liquid and 50,000 *E. bovis* oocysts were added to the 50 ml syringes (N = 10), and were processed similarly to and simultaneously with samples containing soil.

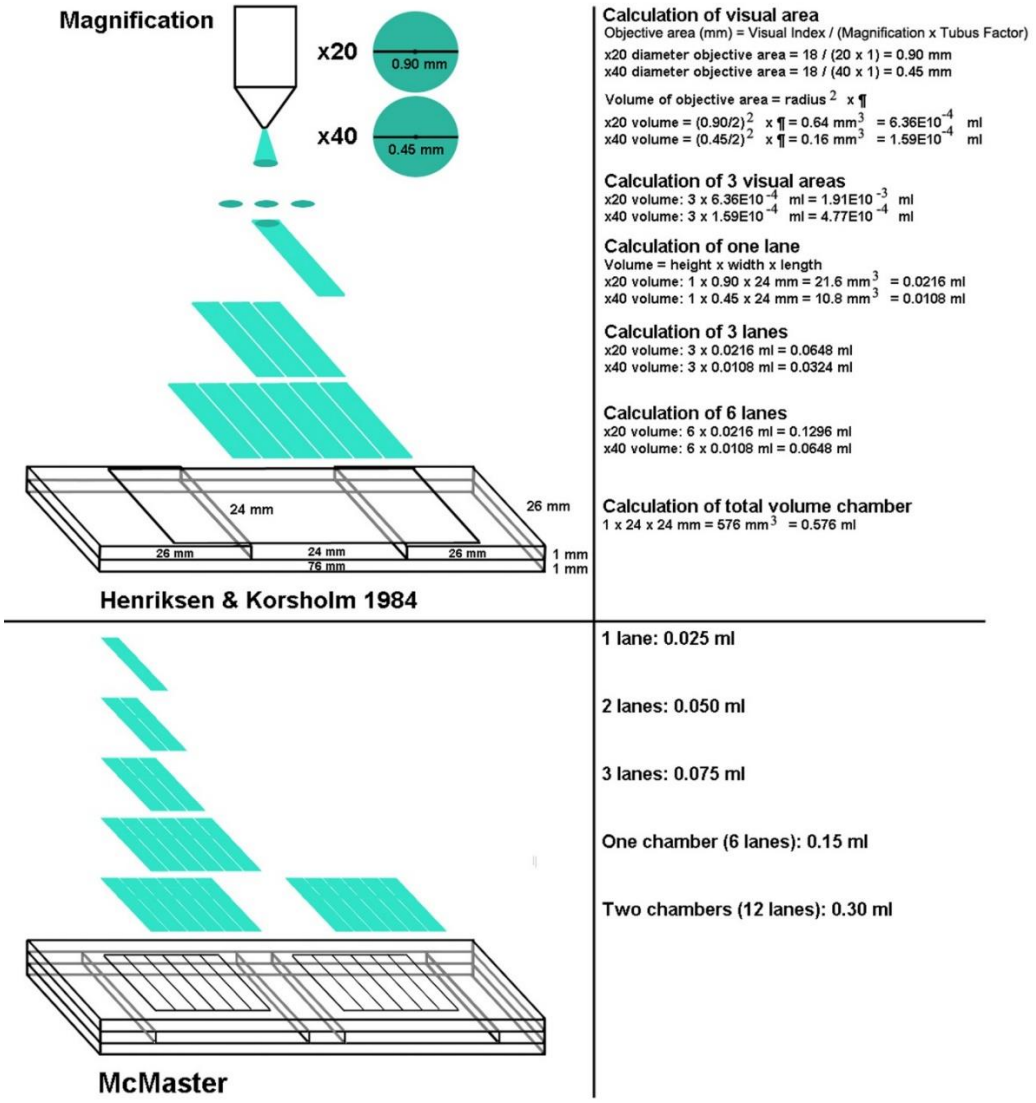

Figure 2. Calculation of the volume examined. Microscope settings and dimensions of the reading chamber are shown

Effect of non-gentle handling. Soil samples were spiked with 17-month-old oocysts at the time of the experiment, using a protocol identical to that described above. At step 3 (Figure 1), the tube was shaken hard 0, 1, 5, or 10 times. Following this step, the protocol was followed as described. Ten repetitions were made for each group.

Statistical methods. Comparison of recovered oocysts in tubes with or without soil, as well as differences between 0 and 1, 5, or 10 shakes, were compared using the Student's t-test. The differences between the groups were examined using the chi-square test. R version 3.0.2. (The R Foundation for Statistical Computing) was used for statistical computations.

Results

Recovery of oocysts with and without soil. In the tubes without soil, 23,109 ± 4,842.7 (45.1 ± 5.9%, 95% CL) oocysts were recovered from the original spiked oocysts.

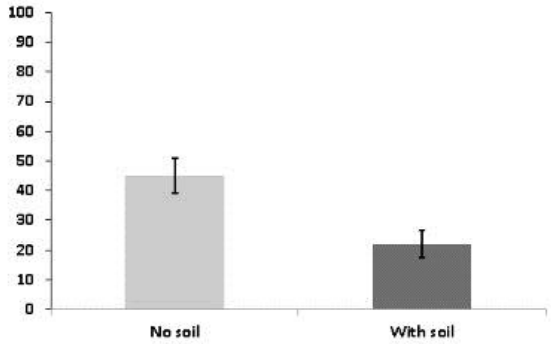

Figure 3. Percentage recovery (%) of oocysts from spiked (with 50,000 *E. bovis* oocysts) flotation liquid and 10 g soil samples using the gentle flotation method. Error bars: 95% confidence limits

In tubes that contained soil, 11,236 ± 5,398.3 (22.0 ± 4.6%, 95% CL, $P < 0.001$) oocysts were recovered (Figure 3). No oocysts were detected in the negative controls.

Effect of non-gentle handling on oocyst recovery. Gentle handling using the protocol gave a 5.6 ± 2.2%,

95% CL recovery rate of the oocysts. A single shake was enough to reduce the recovery rate by almost 50%; to 2.4% ± 1.3, 95% CL. Further shaking, for 5 or 10 shakes, reduced recovery to 1.6% (Figure 4).

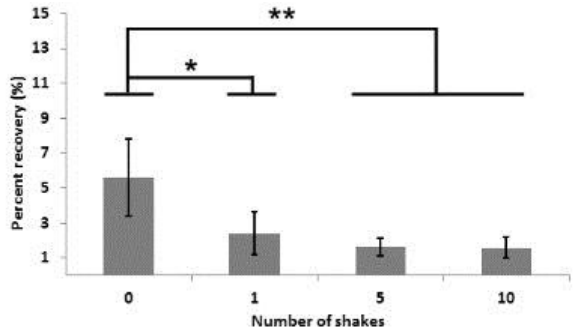

Figure 4. Percentage recovery (%) of oocysts from spiked (with 50,000 *E. bovis* oocysts) soil samples, using the gentle flotation method, after applying 0, 1, 5, or 10 hard shakes of the syringe. * = P < 0.05, ** = P < 0.01. Error bars: 95% confidence limits

Although there were no differences between the groups overall (X2 = 73, P = 0.43), comparing the reduction in oocyst counts between the group with no shaking and the groups exposed to shaking 1, 5, or 10 times, a significant reduction was observed in the recovery of oocysts (P = 0.03, P = 0.01, and P = 0.01 respectively).

Discussion

The method described here for isolating *E. bovis* oocysts was developed as a response to testing different protocols. Large losses of oocysts were observed at the sieving and centrifugation steps using the protocol described by Mejer *et al*. (2000). The method was developed for the recovery of oocysts from soil which considered the oocysts as akin to 'eggs in a basket full of rocks'. The centrifugation steps were removed and sieving was reduced to a piece of gauze, which did not significantly reduce the recovery of oocysts (data not shown). The transfer steps were reduced to two to avoid losses in liquids and losses due to the possible adhesion of oocysts to surfaces. This method has been described in brief previously (Lassen *et al*., 2013; Lassen *et al*., 2014). In this paper, the method is described in detail, including all mechanical steps, and the analytical limitations of the method. The experimental setup in this study is similar to that described by Lassen *et al*. 2013, spiking the soil with 50,000 oocysts, to ensure that the variation could be adequately compared.

A study by Lélu *et al*. (2011) compared different protocols for isolating *Toxoplasma gondii* oocysts and showed that dispersion solutions commonly used in protocols did not perform better than water. The dispersion of the soil was skipped in the described method and the flotation liquid was added directly. Incubating overnight in 2% (w/v) H_2SO_4 to dissolve organic matter and disperse the soil has been described as having the potential to release oocysts (Lélu *et al*., 2011). However, this step was not included in the protocol, in order to shorten the processing time for each sample. As only half the recovery rate for samples containing soil compared with those without soil was observed, the release of oocysts from the soil is a point where this method could be further improved, at the expense of a longer processing time. The methods evaluated by Lélu *et al.* (2011) found that the best protocol tested could recover 18% of 100,000 22-month-old *Toxoplasma* oocysts which had been spiked into 10 g soil samples. Assuming that recovery of *T. gondii* and *E. bovis* oocysts are comparable, this protocol performed slightly better (22%). As recovery rates of oocysts appear to decrease with the age of the oocysts (Lélu *et al*., 2011), the higher recovery rates in this study may be partly attributed to the fact that the oocysts used for testing the recovery rates were only 12-months-old.

Soil composition, and particularly the proportion of sand, appears to affect the recovery rate of *T. gondii* oocysts (Lélu *et al*., 2011), and is also likely to affect the recovery rate of *Eimeria* oocysts. Of the tested soils, the soil composition most closely relating to this study was 97% sand and 3% organic matter. In that soil, less than 20% of the *Toxoplasma* oocysts were recovered, whereas soils with only 30% sand allowed the recovery of more than 30% of the oocysts. This seems supportive of the theory that mechanical handling of the mixture of sand, oocysts, and liquid exerts considerable physical stress on the sample, possibly resulting in loss of oocysts in this type of diagnostics. This was tested in the second experiment, which showed that the effect of sudden movements is enough to significantly reduce the number of oocysts recovered. Although the evidence presented supports the hypothesis of reduced recovery rates due to non-gentle handling, the recovery of oocysts without shaking was lower (5.6%) than the initial testing of the recovery rates (22%). The oocysts were older (17 months) in the experiment investigating shaking than in the experiment evaluating recovery rates, but this is not likely to explain all the variation in recovery. It is more likely that the difference could be attributed to the fact that different persons performed the two experiments.

This method's theoretical analytical sensitivity can be calculated to be 15 oocysts per gram of soil, which could be improved by adding more steps. For example, FLOTAC reading chambers might improve sensitivity, as has been shown for other flotation methods (Cringoli, 2006; Schnyder *et al*., 2011). Methods for gently releasing the oocysts from the soil, such as aeration from below a soil in a liquid, may also improve the success of recovery, but would be technically challenging.

The described method is likely to have practical applications in field testing of environmental contamination with *Eimeria* and potentially other oocysts. Further testing under natural conditions and comparison with other methods extracting parasitic oocysts is recommended.

Conclusion

This simple, inexpensive, and fast method for recovery of *Eimeria* oocysts performed equally well to previously tested methods for oocyst recovery. Gentle handling of soil samples prior to oocyst isolation is important.

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References

Cringoli, G. 2006. FLOTAC, a novel apparatus for a multivalent faecal egg count technique. – Parassitologia, Sep, 48(3), 381–384.

Durant, J.F., Irenge, L.M., Fogt-Wyrwas, R., Dumont, C., Doucet, J.P., Mignon, B., Losson, B., Gala, J.L. 2012. Duplex quantitative real-time PCR assay for the detection and discrimination of the eggs of *Toxocara canis* and *Toxocara cati* (Nematoda, Ascaridoidea) in soil and fecal samples. – Parasit Vectors, Dec 7, 5, 288.

Henriksen, S., Korsholm, H. 1984. Parasitologisk undersøgelse af fæcesprøver. Konstruktion og anvendelse af et enkelt opbygget tællekammer. – Dansk Vet Tidskr, 67, 1193–1196.

Jenkins, M.B., Bowman, D.D., Fogarty, E.A., Ghiorse, W.C. 2002. *Cryptosporidium parvum* oocyst inactivation in three soil types at various temperatures and water potentials. – Soil Biol. Biochem., 34(8), 1101–1109.

Kato, S., Bowman, D.D. 2002. Using flow cytometry to determine the viability of *Cryptosporidium parvum* oocysts extracted from spiked environmental samples in chambers. – Parasitol. Res., 88, 326–331.

Mejer, H., Wendt, S., Thomsen, L.E., Roepstorff, A., Hindsbo, O. 2000. Nose-rings and transmission of helminth parasites in outdoor pigs. – Acta Vet. Scand., 41(2), 153–165.

Koken, E., Darnault, C.J., Jacobson, A.R., Powelson, D., Hendrickson, W. 2013. Quantification of *Cryptosporidium parvum* in natural soil matrices and soil solutions using qPCR. – J. Microbiol. Methods, Feb 15, 92(2), 135–144.

Lassen, B., Lepik, T., Bangoura, B. 2013. Persistence of *Eimeria bovis* in soil. – Parasitol. Res., 112, 2481–2486.

Lassen, B., Lepik, T., Järvis, T. 2014. Seasonal recovery of *Eimeria* oocysts from soil on naturally contaminated pastures. – Parasitol. Res. Mar, 113(3), 993–999.

Lélu, M., Gilot-Fromont, E., Aubert, D., Richaume, A., Afonso, E., Dupuis, E., Gotteland, C., Marnef, F., Poulle, M.-L., Dumètre, A., Thulliez, P., Dardé, M.-L., Villena, I. 2011. Development of sensitive method for *Toxoplasma gondii* oocyst extraction in soil. – Vet Parasitol., 183, 59–67.

Ramirez, N.E., Sreevatsan, S. 2006. Development of a sensitive detection system for *Cryptosporidium* in environmental samples. – Vet. Parasitol., 136, 201–213.

Rehman, T.U., Khan, M.N., Sajid, M.S., Abbas, R.Z., Arshad, M., Iqbal, Z., Iqbal, A. 2012. Epidemiology of *Eimeria* and associated risk factors in cattle of district Toba Tek Singh, Pakistan. – Parasitol. Res., 108(5), 1171–1177.

Schnyder, M., Maurelli, M.P., Morgoglione, M.E., Kohler, L., Deplazes, P., Torgerson, P., Cringoli, G., Rinaldi, L. 2011. Comparison of faecal techniques including FLOTAC for copromicroscopic detection of first stage larvae of *Angiostrongylus vasorum*. – Parasitol. Res. Jul; 109(1), 63–69.

Svensson, C. 1997. The survival and transmission of oocysts of *Eimeria alabamensis* in hay. – Vet. Parasitol., 69, 211–218.

Tavalla, M., Oormazdi, H., Akhlaghi, L., Shojaee, S., Razmjou, E., Hadighi, R., Meamar, A. 2013. Genotyping of *Toxoplasma gondii* Isolates from Soil Samples in Tehran, Iran. – Iran J. Parasitol. Apr; 8(2), 227–233.