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Simplifying the process of extracting intestinal parasite eggs from archaeological sediment samples: A comparative study of the efficacy of widely-used disaggregation techniques

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ABSTRACT

Some scientific techniques are widely used because they work satisfactorily, but they may not be the cheapest, fastest or most efficient method possible. Here we assess the widely used methods for disaggregating archaeological latrine sediments, where solid soils are converted to aqueous suspension prior to microscopic analysis for ancient parasite eggs. It has been noted that there is great variability in protocols described in the published literature. We have used samples from a medieval latrine in Cyprus and a cesspool from Israel containing roundworm eggs to evaluate in a pilot study whether there appears to be distinct advantages to any of the standard protocols. The results suggest that there is very little difference in the efficacy whether disaggregation is performed using traditional 0.5% trisodium phosphate or simple distilled water, whether the process lasts 72 h or just 1 h, or whether sonication is added to the process. While a larger sample size would allow a more robust statistical analysis, this pilot study provides no evidence to suggest the long disaggregation periods, expensive chemicals, or sonication steps leads to any better disaggregation in latrine sediments than using distilled water for just 1 h.

1. Introduction

In their landmark study from over 50 years ago, Callen and Cameron (1960) described having identified the eggs of intestinal parasitic worms (*Diphyllobothrium* sp.) preserved in a coprolite. While no dimensions or illustrations of these eggs were included in their paper, they did describe the eggs as oval and possessing an operculum so it is quite likely they were correct. 'When received, the coprolites looked like lumps of sand and dust, so that the first step was to reconstitute this desiccated material to obtain what might have been its original texture and appearance as far as possible. Success was ultimately obtained by soaking it for 72 h in a 0.5% aqueous solution of sodium triphosphate', followed by microscopy (Callen and Cameron, 1960; Bryant and Dean, 2006). Their paper did not mention what other solutions they may have tried in order to disaggregate the coprolites, but just stated that sodium triphosphate was successful. One might guess that they tried water initially, but the paper does not actually state that.

Since that time, disaggregation of archaeological fecal specimens with trisodium phosphate for several days has become a widely practiced technique by paleoparasitologists, prior to the application of techniques such as microsieves, flotation or sedimentation for separating out the parasites eggs from the disaggregate fluid, and then microscopy (Bouchet et al., 2003; Fugassa et al., 2006; Jiménez et al., 2012; Reinhard et al., 1986; Warnock and Reinhard, 1992). Whilst in the majority of published studies the samples are rehydrated for 72 h as Callen and Cameron did (Fernandes et al., 2005; Rocha et al., 2006; Mitchell and Tepper, 2007), some studies rehydrate the samples for 48 h (Bathurst, 2005), or for multiple weeks (Bouchet et al., 2001; Han et al., 2003; Shin et al., 2009). Additionally, some studies also employ sonication of the samples as an additional step after their rehydration to facilitate the disaggregation process (Bouchet et al., 2002, 2001).

Although it has been demonstrated that trisodium phosphate is an effective medium with which to rehydrate and disaggregate coprolites, sediments or even dehydrated tissues (Fry, 1977; Reinhard et al., 1986), to our knowledge it has never been proven whether rehydration with trisodium phosphate is actually a necessary step when analysing soil sediments from latrines. As far as we are aware no one has published a comparative study to show to whether rehydration and disaggregation with trisodium phosphate is really required when latrine and cesspool sediment samples are analysed instead of coprolites, or whether the time period of rehydration affects the outcome of the analysis in latrine soils. Similarly, the efficacy of the use of sonication to improving disaggregation has not been proven in a systematic manner in a publication.

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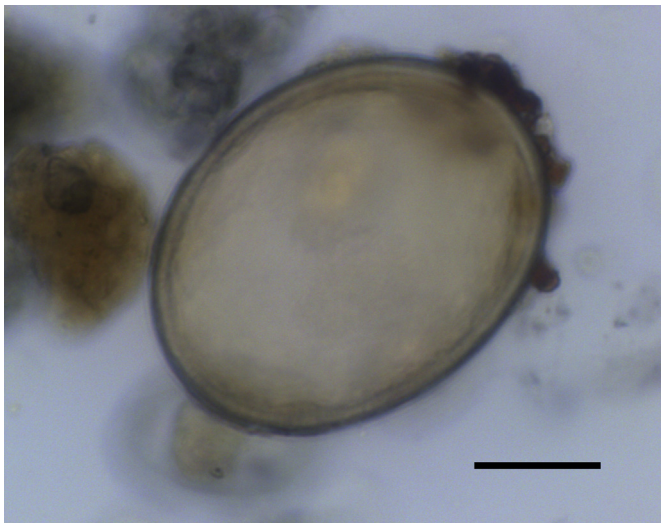

Fig. 1. Decorticated roundworm egg (*Ascaris lumbricoides*) from the 13th century latrine in the crusader Kingdom of Jerusalem. Dimensions 67 μm × 44 μm. Scale bar measures 20 μm.

The aim of this study is to investigate whether the disaggregation medium, the length of time undergoing rehydration or the use of sonication after rehydration have much effect on the number of parasite eggs identified in latrine and cesspool sediments. If these steps can be simplified, it would not only speed up the process of ancient parasite research, but also reduce the costs of analysis.

2. Material

The samples selected for the present study came from a 13th century cesspool in Acre, Israel and from a 12th century latrine in Cyprus. The cesspool was located in the domestic quarter of medieval Acre and was excavated in 2007 by the Israel Antiquities Authority, under Danny Syon. An AMS radiocarbon dating of an animal bone fragment suggests that with 95% probability the cesspool was in use between 1265 and 1303AD, during the crusader period. The paleoparasitological analysis of the cesspool revealed the presence of eggs of *Ascaris lumbricoides* (roundworm) and *Diphyllobothrium latum* (fish tapeworm) (Mitchell et al., 2011). The latrine studied for this analysis is located in the castle of Saranda Kolones, in Paphos, Cyprus and was excavated intermittently between 1966 and 1985 by A.H. Megaw under the auspices of the British School at Athens and of Dumbarton Oaks. The castle was built sometime after 1191 and was destroyed and abandoned after a severe earthquake in 1222AD. The brief period of occupation of the castle allows for a narrow dating of the use of the latrine, in the late 12th and early 13th century AD. During the paleoparasitological analysis of the latrine, eggs of *A. lumbricoides* (roundworm) and *Trichuris trichiura* (whipworm) were identified (Anastasiou and Mitchell, 2013). These two toilet facilities were chosen for the study as they both contained roundworm eggs (Fig. 1), whose numbers could be counted using the same technique. Only the roundworm eggs were counted for this study and not the other species present in each latrine, as roundworm was the species found at both sites. Two independent sites were tested to ensure that the findings from one were reproducible in different archaeological contexts.

2.1. Methods

A total of 12 1 g samples of dried soil sediment from both latrine and cesspool were analysed. Firstly, samples from Acre and from Saranda Kolones were disaggregated by adding 5 ml of a 0.5% aqueous solution of trisodium phosphate for 1 h, 24 h and 72 h, with intermittent gently shaking and stirring with a glass rod. Similarly, 1 g samples from the same part of each latrine were disaggregated in 5 ml of distilled water for the same time periods. After their rehydration, half the samples (six) were also processed for 30 min in an ultrasonicator (the Clifton range MU-8). Table 1 summarises the different approaches followed in the analysis of the samples from Acre and Saranda Kolones.

There are a number of effective methods with which to identify parasite eggs in disaggregated samples. Our method of choice is the sequential micro-sieve method of Bouchet et al. (2003). In order to separate the parasite eggs from the soil components, the samples were processed with a column of micro-sieves (300 μm, 160 μm and 20 μm). Following this, the material on the 20 μm sieve was placed in a 15 ml tube. One lycopodium spore tablet (batch number 938934, $x = 10{,}679$ spores/tablet (Stockmarr, 1971)) was added in the material. This was then treated with 30% hydrochloric acid in order to dissolve the lycopodium tablet releasing the 10,679 spores from the tablet into the material. The use of exotic particles such as lycopodium spores or eucalyptus pollen grains to calculate concentration values of an element of interest, derives from palynology, where the technique is widely used to calculate pollen concentrations (Sobolik, 1998). The number of spores in each tablet is standardised and stated by the manufacturer. In palaeoparasitology, the addition of a known number of exotic spores to the sample allows for an accurate calculation of the number of eggs/g of soil, by calculating the ratio of eggs to the known number of spores (Warnock and Reinhard, 1992).

In this study the lycopodium spore tablet was added after the sieving of the material. Due to the size of the lycopodium spores (25–40 μm) adding the tablet after sieving the samples does not affect the number of spores that end up in the material that will be microscopically examined. Even if the tablet had been added in the original 1 g of soil, after the sieving of the material all 10,679 lycopodium spores contained in the tablet would have been collected in the 20 μm micro-sieve because their size allows them to pass from the micro-sieves of the 300 μm and 160 μm, but not through the micro-sieve of the 20 μm. Moreover, the use of micro-sieves does not affect the calculation of the concentration of eggs/g of soil, because all the eggs present in the gram of soil as well as all the lycopodium spores are concentrated on the 20 μm micro-sieve that will be microscopically examined. Therefore the ratio of lycopodium spores to parasite eggs remains unchanged. The formula used for the calculation of the number of eggs/g of soil in the present analysis is as follows:

$$\frac{\text{Eggs counted}}{\text{Spores counted}} \times \frac{10,679 \text{ spores}}{\text{Sediment weight}} = \text{Parasite eggs/g}$$

During the microscopic examination twenty slides per sample were analysed and all the *A. lumbricoides* eggs and all the lycopodium spores present in each slide were counted. The number of eggs and spores counted in all 20 slides was then used to calculate the concentration of the parasite eggs/g of soil.

2.2. How to compare groups

In order to investigate whether using different rehydration media, different time periods of rehydration, and sonication has any effect on the disaggregation level of the samples and on the results of the paleoparasitological analysis, the coefficient of variation (descriptive statistics) was calculated for the 12 arms of the study for each archaeological site. For statistical reasons it is only possible to prove beyond doubt that no difference exist between these groups if at least six 1 g samples were processed for each of the 12 arms of the study, for both latrines. Not only would this require a much larger amount of soil than was available to us (at least 144