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Abstract

The aim of this research was to determine the species of intestinal parasite present in a Roman Imperial period population in Asia Minor, and to use this information to improve our understanding of health in the eastern Mediterranean region in Roman times. We analyzed five samples from the latrines of the Roman bath complex at Sagalassos, Turkey. Fecal biomarker analysis using 5β-stanols has indicated the feces were of human origin. The eggs of roundworm (*Ascaris*) were identified in all five samples using microscopy, and the cysts of the protozoan *Giardia duodenalis* (which causes dysentery) were identified multiple times in one sample using ELISA. The positive *G. duodenalis* result at Sagalassos is particularly important as it represents the earliest reliable evidence for this parasite in the Old World (i.e. outside the Americas). As both these species of parasite are spread through the contamination of food and water by fecal material, their presence implies that Roman sanitation technologies such as latrines and public baths did not break the cycle of reinfection in this population. We then discuss the evidence for roundworm in the writings of the Roman physician Galen, who came from Pergamon, a town in the same region as Sagalassos.

1. Introduction

Sagalassos in southwestern Turkey was the most important urban settlement in the region of Pisidia during the Roman Imperial period (Figure 1). It was incorporated in the Roman Empire in 25 BC and remained prosperous into late antiquity. Its high altitude (1400-1600m above sea level), fertile territory, access to fresh and salt water, and location along one of the key roads north from the Mediterranean harbors of Pamphylia, all contributed to its

establishment as a regional economic center based around the local ceramic industry (Sagalassos Red Slip Ware) and the production of cash crops such as grains and olives (Fuller et al., 2012; Poblome, 2015).

While a good number of Roman period sites have been analysed for intestinal parasites, some parts of the Roman Empire have had no analysis whatsoever (Mitchell, 2016). These include central and western parts of North Africa (Libya, Tunisia, Morocco), the Iberian Peninsula (Spain and Portugal), the Mediterranean islands, and Asia Minor (Turkey). Indeed, there has been no parasite analysis of any archaeological sites from Turkey published, from any time period in history. Given the absence of data from Turkey, Sagalassos is an important site for understanding intestinal health in Roman Asia Minor.

The aim of this study is to determine the types of intestinal parasites that infected those people using the latrines at the Roman baths at Sagalassos in order to see how this region compares with other parts of the Roman Empire at that time. This will not only provide insight into the health of the inhabitants of Sagalassos, but also allow us to better understand their diet, cooking habits, level of sanitation, and agricultural practices. One of the key physicians of the classical world was Galen of Pergamon (c.130-c.210 AD), and Pergamon was a town in the same region as Sagalassos. We will use the evidence for parasites in Galen's medical texts to understand what people in the Roman period thought of these diseases.

2. Materials

During excavation of the vaulted rooms supporting the western parts of the imperial bath complex, a communal latrine was identified (Figure 2). The actual toilet infrastructure

had been removed in antiquity, but sufficient traces were left to positively identify Room 4 as a latrine. These include the holes in the mortared brick walls for affixing toilet seats, and the partially preserved sewage channels under where the toilet seats would have been. The sewage channels collected the excrement and drained it into the sewer opening in the north-west corner of the room. Water was recycled from the baths on the level above and entered the room through a channel in the eastern wall (Figure 3). This washed the feces from under each seat along the sewage channels. In late antiquity the toilets seats were removed from the latrine and the room was used to collect urban refuse, and for the composting of animal manure (Baeten et al., 2012).

At the time of excavation multiple sediment samples were taken from Room 4 (samples A-E). The samples were analyzed at the University of Leuven (Belgium) using gas chromatography mass spectrometry (GCMS), atomic absorption spectroscopy (AAS) and microscopy to study fecal biomarkers, calcium concentration, and presence of plant macrofossils, pollen, and fungal spores. Fecal biomarkers can differentiate fecal material from different animal species. If the fecal material is omnivore (e.g. human) in origin then coprostanol and epicoprostanol will dominate, while in feces of herbivore origin 5β-stigmastanol and 5β-epistigmastanol will dominate (Linseele et al., 2013; Shillito et al., 2011). For the Sagalassos latrine the fecal biomarker results showed that the sediment above ground level in the vaulted room (sample A) represented decomposed animal dung from herbivores, with coprostanol: 5β-stigmastanol ratios around 0.05. In contrast, sediment in the sewage channels (samples C and E) represent decomposed omnivore feces with coprostanol: 5β-stigmastanol ratios within the range 1.0 to 3.45, compatible with a human origin (Baeten et al., 2012). Calibrated AMS radiocarbon dating of charcoal fragments from four samples

suggests the fecal material dates from the 2nd-5th centuries AD (Baeten et al., 2012). Five samples with fecal biomarkers indicating human feces, and four samples with biomarkers indicating herbivore feces, were then sent to Cambridge for parasite analysis.

3. Methods

In order to analyze the samples for intestinal parasites, 0.2 grams of dry latrine sediment was suspended in 5ml 0.5% aqueous solution of trisodium phosphate until disaggregated. This suspension was passed through a series of stacked micro-sieves of mesh size 300μm, 160μm, and 20μm. Most species of intestinal helminth that infect humans in this region produce eggs with dimensions between 30μm and 150μm, so the parasite eggs within the samples would be trapped on the mesh of the 20μm sieve (Bouchet et al., 2003). After washing this material from the 20μm sieve the resulting suspension was centrifuged to concentrate the sample, the supernatant discarded, and glycerol added before mounting on slides. Slides were viewed under a light microscope at 400x magnification to identify and quantify the parasite eggs present. Eggs were identified by shape, size, color, and special features as described in standard parasitology sources (Garcia, 2009; Gunn and Pitt, 2012). The entire 0.2g sample was viewed on a series of slides. Egg counts per gram of soil were calculated by counting the number of eggs observed across all slides for a given sample, and then multiplying this number by 5.

Disaggregated sediment that passed through the 20μm sieve was collected and analyzed for the presence of protozoa that cause dysentery, specifically *Entamoeba histolytica*, *Giardia duodenalis*, and *Cryptosporidium parvum*. Eight subsamples from each latrine sample were tested with Enzyme-Linked Immunosorbent Assay (ELISA) test kits specifically designed by

Techlab$^©$ to detect the cysts of these organisms, which range in size from 2-20µm. They employ a monoclonal antibody-peroxidase conjugate specific for proteins uniquely secreted by these organisms, with reported 100% sensitivity and 99.8-100% specificity (Sharp et al., 2001). The ELISA reader used was a BioTek Synergy HT Multi-Mode Microplate Reader, with measurements taken at a wavelength of 450nm. This ELISA analysis was then repeated at a later date to ensure reproducibility of our results.

4. Results

4.1 Helminths

All five latrine sediment samples were positive for roundworm (*Ascaris sp.*) eggs. Roundworm was identified by its oval shape, color, and distinct wall (Figure 4). Although intact *Ascaris sp.* eggs normally have a thick, mammillated outer layer, the eggs observed in this study were decorticated. This is common in archaeologically recovered eggs, as taphonomic processes frequently interfere with its preservation. The egg dimensions and counts per gram are given in table 1. As the eggs were decorticated, the dimensions are slightly smaller than we would expect for eggs that had a intact mammillated coat. They are consistent with fertilized human *Ascaris* egg dimensions reported in standard parasitology sources (Garcia, 2009; Gunn and Pitt, 2012). While every sample tested was positive for roundworm eggs, it should be noted that the concentration of eggs in the latrine sediment was not high.

In addition, one single egg of *Dicrocoelium sp.* (lancet liver fluke) was identified in sample 1268 (Figure 5). This soil sample was positioned closest of the five to the herbivore dung layer. The dimensions fall with the normal range for *D. dentriticum* (Le Bailly and Bouchet, 2010).

Since *Dicrocoelium sp.* is a common parasite of ruminant animals, and only one egg was found in the human fecal layer, it is likely that it may have percolated down through the soil from the animal dung layer into the layer of human feces below. Analysis of the four herbivore layer samples found no evidence for roundworm eggs in any of them.

4.2 Protozoa

ELISA analysis tested for the presence of the protozoal parasites *E. histolytica, G. duodenalis* and *C. parvum* using Techlab$^©$ test kits. We performed this analysis twice using different kits on dates several months apart in order to ensure reproducibility of our results. On our first analysis of the human layer samples, sample 1275 was positive for *G. duodenalis* in 2 samples out of 8 tested (Figure 6). For a positive result using the Giardia II test kit, the ELISA absorbance reading must be greater than 0.150 and the samples in question gave absorbance readings of 0.263 and 1.119. On the repeat analysis, we tested the sample in 16 wells, in the center of the plate to avoid the edge. In this analysis 7 wells were positive (absorbance readings of 0.678, 0.827, 0.218, 0.615, 0.692, 1.241, 0.194).

The same sample (1275) was also positive for *C.* parvum in the initial test (1 sample out of 8 tested). The threshold for a positive *C. parvum* result is also 0.150 and the positive sample gave an ELISA absorbance reading of 0.206. On repeat testing, the result was negative in all 16 wells used. As only one well was positive, and this was not reproduced in the second test, and since *C. parvum* has never before been identified in Old World samples, we are interpreting this *C. parvum* result cautiously in case it is a false positive. All the latrine samples were negative for *E. histolytica.*

We also analysed the four herbivore layer samples with the *Giardia duodenalis* ELISA test kit. All four samples were negative.

5. Discussion

Analysis of the latrine samples from the sewage channel under the Roman baths complex at Sagalassos identified good evidence for roundworm and *Giardia duodenalis,* which is a protozoan that causes dysentery in humans and some mammals. While the sediment layers above the original floor level of the latrine have been shown to be comprised of herbivore dung, the fecal biomarkers for sediment from the sewage channel indicated feces of human origin (Baeten et al., 2012). Out of five human fecal samples we only noted one egg of a species that commonly infects farm animals (*Dicrocoelium sp.*). This was found in the human fecal sample that was closest in distance to the herbivore dung layer. The presence of the *Dicrocoelium sp.* egg would be consistent with the downward migration of fecal biomarkers that was noted in the upper soil layers of the sewage channel (Baeten et al., 2012). Analysis of samples from the herbivore layer were negative for roundworm eggs on microscopy, and negative for *Giardia duodenalis* on ELISA analysis. This would suggest that the parasites we found in the human feces layer indicate genuine infection of the people who used the latrine, and not contamination of the human layer by parasites that percolated downwards from the herbivore layer.

The concentrations of parasite eggs per gram were not high (Table 1), and if we were studying coprolites then this would be interpreted as indicating a light infection with just a few worms. However, as we are studying latrine soil from feces mixed together from many individuals, and not individual coprolites each from a single person, we cannot interpret the

eggs per gram in such a way. For example, it could be that some people using the latrines had heavy infections while most people were not infected at all, so leading to the latrine soil concentration seen here. In some archaeological latrines egg concentration may be low if people disposed of lots of rubbish in the latrine, as well as human feces. However, the archaeological excavations of the latrine did not indicate this practice. In any case, it is unlikely that this would have taken place at Sagalassos when the latrines seats were still in situ and the latrine was used just by those using the public baths.

In order to interpret the significance of these results, we will first describe the intestinal parasites we might expect to find in a Roman period sample from the Middle East, and then discuss why our findings are of particular interest. Previous ancient parasite studies in the Middle East prior to the Roman period have been undertaken in Cyprus, Egypt, Iran, Israel, and Syria. They have identified the presence of roundworm (*Ascaris sp.*), whipworm (*Trichuris trichiura*), beef/pork/Asiatic tapeworm, (*Taenia* sp.), fish tapeworm (*Diphyllobothrium* sp.), liver fluke (*Fasciola* sp.), Lancet liver fluke (*Dicrocoelium sp.*), pinworm (*Enterobius vermicularis*), and bilharzia (*Schistosoma haematobium or S. intercalatum*) (Anastasiou et al., 2014; Cahill et al., 1991; Harter, 2003; Harter et al., 2004; Harter-Lailheugue et al., 2005; Nezamabadi et al., 2013a, 2013b; Witenberg, 1961; Zias et al., 2006).

Studies of Roman period archaeological samples elsewhere in the Roman Empire have identified the intestinal parasites roundworm, whipworm, *Taenia sp.* tapeworm, fish tapeworm, pinworm, lancet liver fluke, *Fasciola sp.* liver fluke, and capillariosis (*Capillaria hepatica*) (De Rouffignac, 1985; Harter, 2003; Heirbaut et al., 2011; Horne, 2002; Jansen and Over, 1966, 1962; Le Bailly and Bouchet, 2006, 2013, 2015; Le Bailly et al., 2010; Mowlavi

et al., 2014; Pike, 1968; Rousset et al., 1996; Searcey et al., 2013; Szidat, 1944; Wilson and Rackham, 1976). Witenberg (1961) claimed to have microscopically identified *E. histolytica* and *G. duodenalis* in Roman period samples from Israel, but because he included no images or descriptions of his findings these results must be considered skeptically. It has been found that whipworm, roundworm and fish tapeworm seem to have had the widest geographic spread across the Roman Empire (Mitchell, 2016).

All five of the Sagalassos latrine samples were positive for roundworm. All the eggs were decorticated, having lost their mammillated coat. This is quite common in archaeological latrine sediments, and is generally due to taphonomy (decay and decomposition). Given that the samples span multiple radiocarbon date ranges between the 2nd – 5th centuries AD, and were recovered from two different loci within the latrine, this would suggest that roundworm was fairly widespread among the inhabitants of Sagalassos, as was the case in the wider Roman Empire. Roundworm is thought to be an heirloom parasite that has been infecting our species throughout our evolution. It seems to have become more common in the last few thousand years as population density increased and when people became sedentary farmers (Mitchell, 2013). Because roundworm is a fecal-oral parasite spread by the contamination of food or water with human fecal material, its presence in every sample at Sagalassos does indicate that human feces had somehow contaminated food to re-infect the population.

G. duodenalis was identified multiple times in both analyses of latrine sample 1275. Until now the earliest examples of this species in the Middle East with a reliable biomolecular diagnosis (ELISA) had dated from the medieval period (Mitchell et al., 2008; Yeh et al., 2015). In Europe the earliest reliable evidence for *G. duodenalis* comes from 7th-9th century

AD Switzerland, and there are a number of positive results from the medieval and post medieval period (Gonçalves et al., 2002; Le Bailly et al., 2008; Le Bailly and Bouchet, 2012; Mitchell, 2015). The parasite has yet to be identified in archaeological samples from Africa or Asia. In consequence, this Roman Period sample is the earliest evidence in the Old World to date by several hundred years, and improves our understanding of the origins of this parasite. Research in the Americas has described feces from the intestines of a number of Andean mummies dating from 1,000 BC – 500 AD to be positive for *G. duodenalis* using both fluorescent antibody microscopy and ELISA (Allison et al., 1999), and one 13th century AD sample from the USA was positive for *G. duodenalis* on ELISA (Gonçalves et al., 2002). Microscopy with fluorescent monoclonal antibody stain has been used to detect *Giardia sp.* to genus level in coprolites from the Americas as early as 2,375-1,525 BC. The coprolites were thought to be human in origin based upon morphology, texture and size of the specimens (Ortega and Bonavia, 2003).

Roman cities were well equipped with public latrines and bathhouses, and regular bathing was a widespread practice among Romans. Latrines were described in Roman sources as including sponges on sticks (taking the function of toilet paper) and basins for hand washing (Jackson, 1988; Taylor, 2005). Roundworm and *G. duodenalis* are fecal-oral parasites, so their presence indicates that human feces had contaminated food or water. This could be explained by a failing to wash hands after defecating or before cooking, by excrement disposal accidentally contaminating food or water sources, or the deliberate use of human feces as agricultural fertilizer (Ziegelbauer et al., 2012). Roman bathwater was not always changed quickly enough to prevent the buildup of grime and cosmetics, and both sick and healthy people sometimes bathed together. There would have been potential for

roundworm and protozoa that cause dysentery to spread in warm, dirty water, especially if people were eating while bathing, as seems to have been common practice (Fagan, 1999). Although Roman cities such as Sagalassos often had robust public sewer systems, few private homes were connected to the public sewers. Instead, waste was collected in cesspits, which were often located near the kitchen for ease of rubbish disposal. However, this would have presented a significant risk of accidental food or water contamination (Jackson, 1988; Scobie, 1986; Taylor, 2005). In addition, collected human excrement was often sold to farmers for use as fertilizer (Taylor, 2005), and the Roman writer Varro makes reference to the practice among farmers of placing their privies above their compost piles (Hooper and Ash, 1934). It is entirely possible that similar practices at Sagalassos contributed to the spread of roundworm and protozoa that can cause dysentery.

Our ELISA analysis for *Cryptosporidium parvum* did have one positive well out of eight for sample 1275 in our initial test, but 0 out of 8 in our retest. In the past *C. parvum* has been found only in pre-Columbian samples from the Americas (Allison et al., 1999; Gonçalves et al., 2003; Morrow and Reinhard, 2016; Ortega and Bonavia, 2003). In view of this, we feel it would be prudent to be cautious about this result. When the ELISA kits were first independently analyzed using modern fecal samples with known infections, the researchers did note one false positive test for *C. parvum* (Sharp et al., 2001). Unless further early samples are found to be positive at other Old World sites in the future, we would not go as far as to argue that this result indicates that *C. parvum* was present in the Roman Empire.

There was no evidence for tapeworm infection at Sagalassos, although beef tapeworm (*T. saginata*), pork tapeworm (*T. solium*)*,* and fish tapeworm (*D. latum*) were all potentially expected as they have been found across the Roman Empire. It is unlikely that this is due to

taphonomy at the site, as beef, pork and fish tapeworm eggs have tough walls comparable to those of roundworm, which was observed in all the samples tested. These tapeworms spread when humans consume the uncooked flesh of infected beef, pork or fish that contain the larval stage of the tapeworm, although thorough cooking will destroy the parasite and prevent infection. Beef, pork, and freshwater fish were all consumed at Sagalassos (Fuller et al., 2012; Van Neer et al., 1997), and preserved fish were imported from as far away as Egypt (Arndt et al., 2003). Therefore, it seems likely that fish and meat were generally cooked properly before consumption, which is furthermore evidenced by analyses of lipid residues from cooking pots from Sagalassos (Kimpe et al., 2005; Romanus et al., 2007). This is an inference which might be explained by the many Roman recipes collected in the *Apicius* (4th-5th century) which called for meat to be cooked twice during preparation (Grocock and Grainger, 2006).

The most famous physician in the Roman Empire at the time these latrines were in use was Aelius Galenus (Hankinson, 2008; Johnston, 2006; Mattern, 2013). Galen was born in Pergamon in northwest Asia Minor, and he lived from c.130 AD to c.210 AD. He was physician to three Roman emperors, Marcus Aurelius (ruled 161-180 AD), Comodus (ruled 180-192 AD) and Septimus Severus (ruled 193-211 AD). These dates correspond to the lower margin of the radiocarbon dates of our samples from the Sagalassos latrines. Pergamon was just 250 miles from Sagalassos, so it is likely that many of the diseases Galen encountered when he learned the art of medicine in Pergamon would have also been present in Sagalassos. Galen wrote a large number of medical texts in ancient Greek during his career, and a number of them mention intestinal worms. He described three types of intestinal worms, and the worms he termed 'teretes' seem to match shape and size of roundworm (*Ascaris*) well (Jirsa and Winiwarter, 2010). Galen thought that intestinal worms were created spontaneously when

putrified matter was affected by heat. The treatments he recommended for worms in his medical texts involved modifying the diet, bloodletting, and medicines that were thought to have a drying and cooling effect on the body in order to return the four humors to a healthy balance. Medicines he recommended in his work *De Simplicium Medicamentorum* for killing intestinal worms included myrrh (a tree resin), cardamom, cumin, mint, Egyptian cabbage, ground peach leaves, artemisia, yellow lupin with honey, the bark of black mulberry, along with other plants that have yet to be identified (Jirsa and Winiwarter, 2010). Interestingly pharmacological research has demonstrated that some of these plants have antihelminthic properties (Githiori et al., 2006; Idris et al., 1982; Khan et al., 2015; Mahmoud et al., 2002; Molefe et al., 2012). Roman herbal treatments for parasites might potentially have had a mitigating effect on the severity of intestinal parasite infections. Therefore it is possible that the low concentration of roundworm eggs, and the absence of whipworm and other species, could in part be due to the use of such medications by the population of Sagalassos.

6. Conclusion

Parasite analysis of five latrine soil samples from Sagalassos has identified evidence for roundworm and the protozoan *G. duodenalis* using microscopy and ELISA. The identification of *G. duodenalis* at Sagalassos is notable for representing the earliest evidence for the species anywhere in the Old World. From the presence of these species, bolstered by consultation of relevant historical sources, we can infer that sanitation conditions in Sagalassos did not prevent reinfection of the population by helminths, despite the availability of a public bath, toilet facilities, and an elaborate urban sewage system. The picture we see at this site is the dominance of parasite species that are spread by dirt and feces.

Bearing in mind we know of ten different species of intestinal parasite that were present in the Roman Empire, we might have expected to see a greater range of parasite species at Sagalassos. This could indicate a regional tradition of thorough cooking of meats and fish before they are eaten, or an environment that did not favor the survival of zoonotic parasites in wild animals of the region. However, the fact that Roman period medical texts from the region recommend treatments now known to have active anti-helminthic components may explain the apparent low number of species and roundworm egg concentrations seen here. This goes to highlight how variable intestinal parasite infection seems have been in the past among different regions and even different members of the same population.

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Sample	Mean egg length in μm	Mean egg width in μm	Eggs per gram
Sample 1268	59.31 (SD 6.40)	48.08 (SD 4.90)	25
Sample 1272	62.04 (SD 2.76)	46.10 (SD 0.94)	15
Sample 1275	62.64 (SD 2.62)	47.13 (SD 2.23)	20
Sample 1291	59.29 (SD 4.35)	42.86 (SD 1.77)	45
Sample 1294	63.67 (SD 5.18)	48.76 (SD 3.03)	10

Note: SD = standard deviation.

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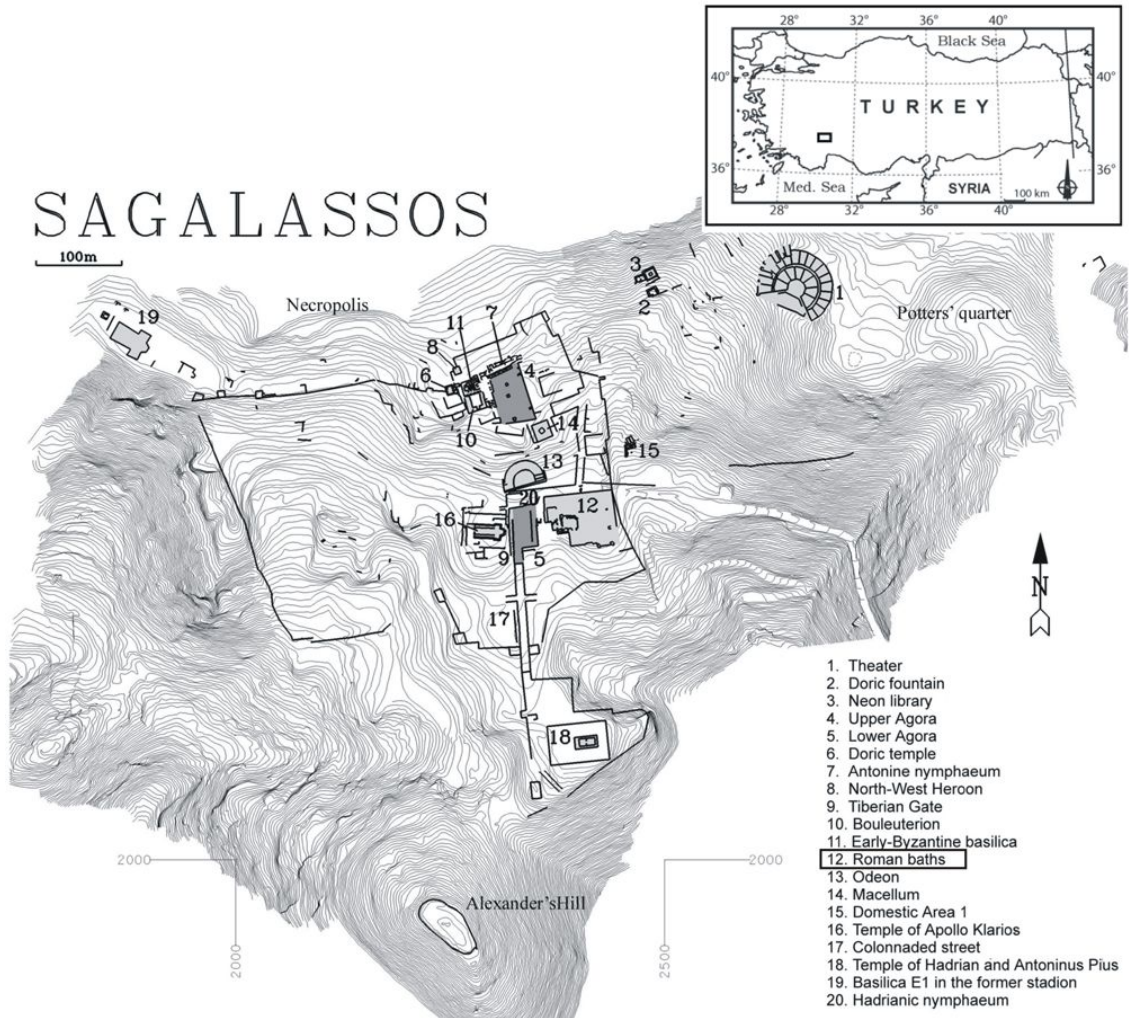
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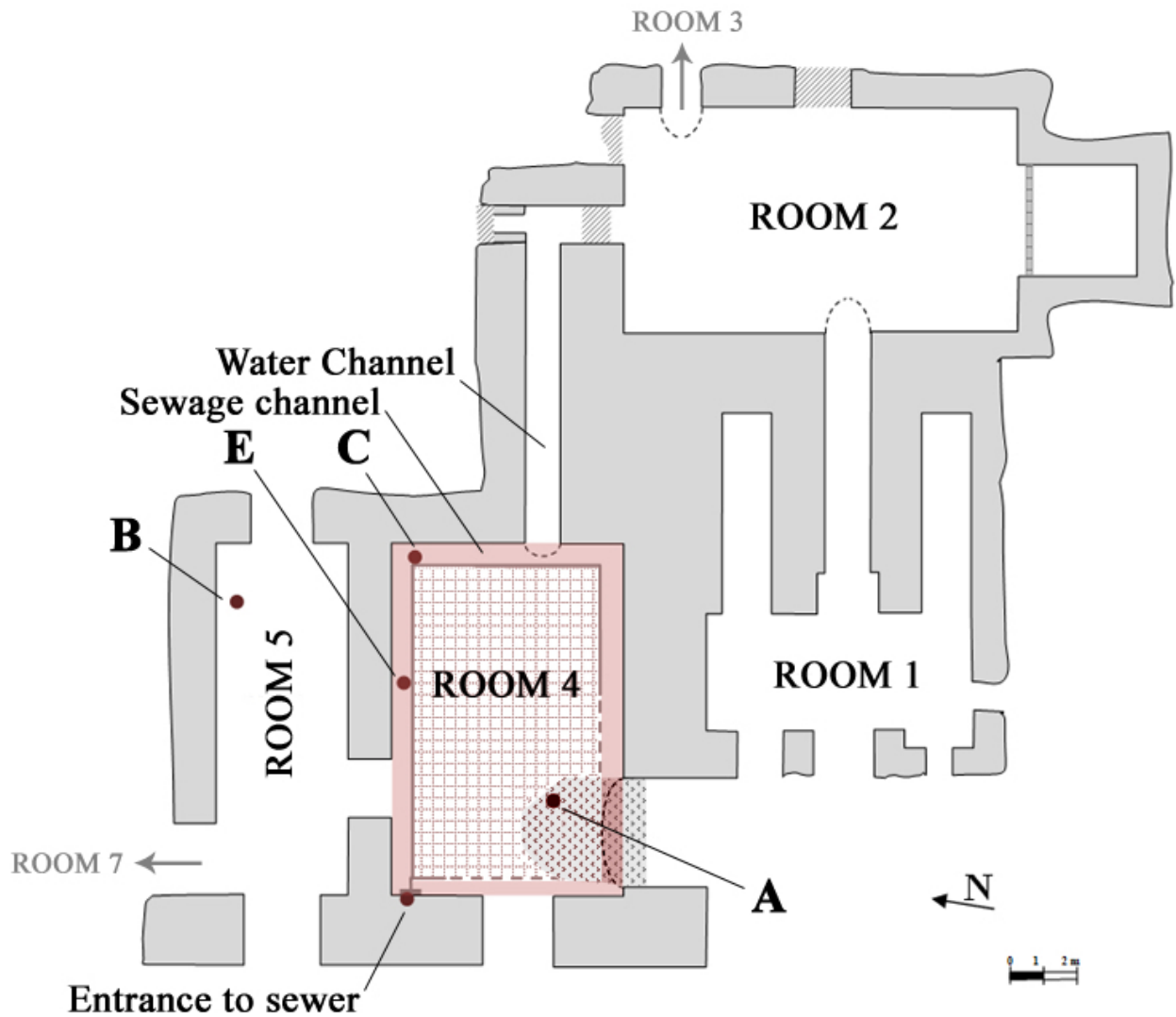
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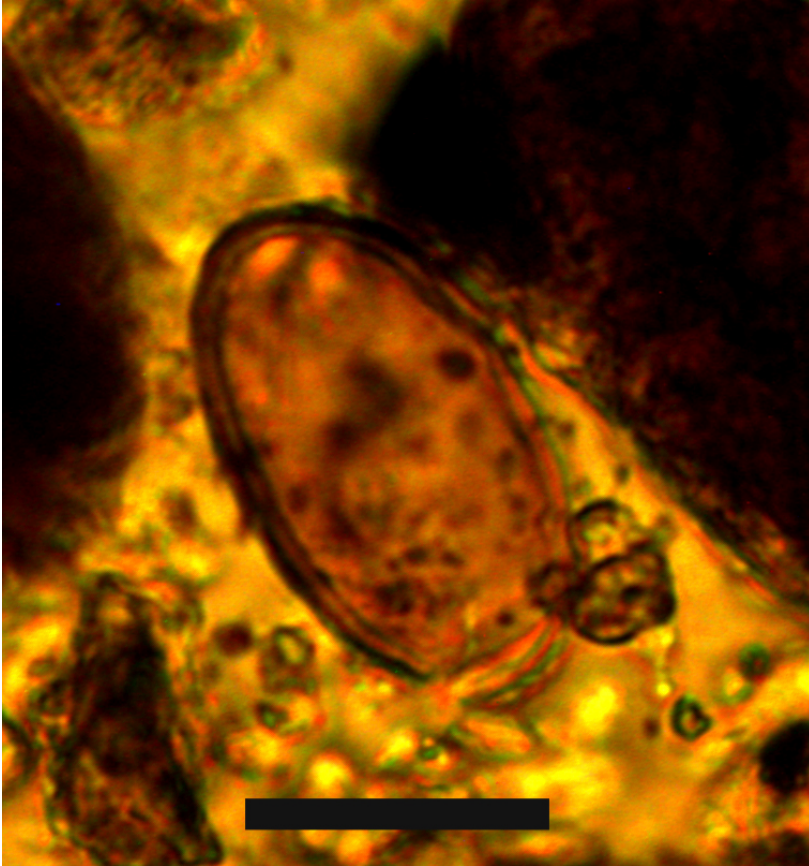
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