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Gastrointestinal parasite burden in 4th-5th c. AD Florence highlighted by microscopy and paleogenetics.

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Abstract

The study of ancient parasites, named paleoparasitology, traditionally focused on microscopic eggs disseminated in past environments and archaeological structures by humans and other animals infested by gastrointestinal parasites. Since the development of paleogenetics in the early 1980s, few paleoparasitological studies have been based on the ancient DNA (aDNA) of parasites, although such studies have clearly proven their utility and reliability. In this paper, we describe our integrative approach for the paleoparasitological study of an ancient population from Florence in Italy, dated to the 4th-5th c. CE. The first stage consisted in the study of sediment samples from the pelvic area of 18 individuals under light microscopy. This allowed us to detect Ascarid-type eggs belonging very probably to the human-infesting roundworm *Ascaris lumbricoides*. Ten subsamples were selected corresponding to five individuals, and we extracted their whole DNA following sediment aDNA protocols. A targeted approach allowed us to detect two nematodes and one trematode aDNA fragments, namely *Ascaris* sp., *Trichuris trichiura*, and *Dicrocoelium dendriticum*. Among the five individuals tested for microscopic eggs and aDNA, three of them showed the remains of eggs (only Ascarid-type), but all of them tested positive to the presence of at least one parasite aDNA. Microscopic diagnosis first guided our research for the selection of promising samples while the targeted aDNA approach significantly improved our knowledge in terms of parasitic diversity and frequency in this population subgroup. These results enabled us to discuss the possible impact of latent parasitism in this past population at the time of an epidemic, as suggested in Florence. In particular, the singular case of *D. dendriticum* detection is discussed in light of the present-day scarcity of genuine human infections. Nevertheless, actual infections are known in the paleoparasitological record, and food habits may have led to false parasitism in this historical context. aDNA leaching from overlying strata may also explain

this detection. This study strongly pleads for a systematic integrative approach combining microscopy and aDNA in paleoparasitology.

Keywords

Paleoparasitology; microscopy; paleogenetics; roundworm; whipworm; lancet liver fluke; bioarchaeology; emergency burial site; Florence

1. Introduction

Paleoparasitology aims to study the natural history of parasites in humans and non-human animals through the recovery of microscopic eggs and cysts laid by ancient helminths and protozoa (Dittmar et al., 2013; Le Bailly and Araujo, 2016). Few aDNA-based studies have been undertaken in this discipline compared with other fields where paleogenetics is commonly applied (Loreille et al., 2001; Madden et al., 2001; Iñiguez et al., 2003, 2006; Aufderheide et al., 2004, 2005; Zink et al., 2006; Liu et al., 2007; Nerlich et al., 2008; Fernandez et al., 2008; Leles et al., 2008, 2014; Costa et al., 2009; Botella et al., 2010; Hawass et al., 2010; Oh et al., 2010; Khairat et al., 2013; Lalremruata et al., 2013; Shin et al., 2013; Wood et al., 2013; Ghul et al., 2014; Matheson et al., 2014; Myskova et al., 2014, Panzer et al., 2014; Novo et al., 2015; ; Côté et al., 2016; Gelabert et al., 2016; Boast et al., 2018; Flammer et al., 2018; Sjøe et al., 2015; 2018; van Dorp et al., 2019; Guedes et al., 2020 ; Sabin et al., 2020). Molecular paleoparasitology studies were recently reviewed by Côté and Le Bailly (2017) and Wood (2018). Similarly, funerary contexts, which may allow for a better evaluation of past infectious dynamics in ancient populations have not been

extensively investigated in comparison with the multitude of studied bio-anthropological records (Harter, 2003; Aufderheide et al., 2004; Hibbs et al., 2011; Jaeger et al., 2014; Marciniak et al., 2016; Anastasiou et al., 2018; Roche et al., 2019; Guedes et al., 2020). In this paper, we focused on the intersection of these two points through the study of five individuals from the emergency burial site discovered under the basement of the Uffizi Gallery in Florence, Italy.

The ancient city of Florence (*Florentia*), Central Italy, was founded by the Romans at the end of the 1st c. BCE and flourished during the Roman Imperial period as a trade centre along the Via Cassia, one of the main roads that departed from Rome. The city was prosperous during the 2nd c. CE, then economically declined in the Late Roman period up to the beginning of the 5th c. CE, when it was besieged by the Goths, led by their chief Radagaisus (De Marinis, 1996; Heather, 2006; Cardini, 2006; Cantini et al. 2007). The Goth army was meanwhile defeated in August 405 CE by the Master-General Stilicho and never managed to penetrate the city. However, the siege had dire consequences for the people of *Florentia* (Davidsohn, 1969).

Archaeological excavations conducted between 2008 and 2014 under the basement of the Uffizi Gallery in Florence led to the discovery of a residual section of what was most likely an extensive burial area. A total of 75 individuals have been exhumed up until now; some from single graves but most of them from 14 multiple graves containing up to ten deceased (Fig. 1). Taphonomical conditions showed that this area corresponded to an emergency burial site dug outside the city walls, possibly in response to an epidemic of still unknown aetiology (Pacciani et al., 2018). Bioanthropological and paleopathological examinations of those remains are still ongoing, but no obvious pathological signs or marks of interpersonal violence were recorded in the field. Minted coins associated with some skeletons indicate that

this site was used somewhere between the second half of the 4th c. and the beginning of the 5th c. CE.

2. Materials and Methods

2.1 Site occupation and geology

The necropolis was located on the edge of an elongated branch of the River Arno (Pallecchi, 2006; Arnoldus, 2007). The soil concerned is thus made up of alternating clayey sands and sandy silts above a gravel base due to frequent alluvial episodes. Specifically, the burials were located in a soil consisting of fine silty and clayey sands in an oxidizing environment. As there are no traces of paleosoils, the soil appears to have been exposed to the elements, with little vegetation. A deep test pit made at the side of the necropolis showed that these sediments continue downwards over a depth of at least three metres.

The area shows no traces of pre-existing use, and seems to have been subject to erosive and sedimentation activities related to seasonal variations in the flow of the river before and after the installation of the necropolis.

To date, it has not been possible to define the extension of the necropolis. The construction of the late medieval quarter in the second half of the 13th century and subsequent looting during the construction of the Uffizi Gallery led to the partial or total removal of some pits. The necropolis appears to be an emergency burial area which was left to the erosion / sediment activities of the river once the need for a burial place was over.

Starting from the 6th- 7th century CE, the whole area southeast of the city seems to have gone through a process of remediation by the creation of an artificial containment embankment (Lelli, 2007; Lelli and Spezi, 2007; Scampoli, 2007).

Between the 11th and 12th centuries, the embankment was raised by the superposition of a massive wall structure, to expand the countryside and acquire new areas destined for use as landfills and / or urban vegetable gardens and agricultural land. This is illustrated by the presence of some dark layers extending, at different levels, into the adjacent area under the building of the Biblioteca Magliabechiana (Nicosia et al, 2012), as well as the discovery of deep subcylindrical garbage pits immediately north of the aforementioned building, attributable to the 13th century (Lelli and Spezi, 2007). These wells or disposal rooms take the form of truncated cone-shaped chambers with dry stone and brick walls. Some were quite deep, and affected the underlying stratigraphy, including the burials, down to the sandy silts. They were used for the collection of sewage which periodically had to be emptied of solid components. The liquid components were constantly present in the well, and could infiltrate the surrounding soil with fine suspended materials, enriching it with organic components and thus also altering its chemical characteristics. In effect, the bone remains of the individuals near the wells appeared to be altered, with chromatic variation tending towards dark brown, and a more fragile consistency than those found further from the wells. The variation in pH, ranging from slightly acidic to basic, may be induced by the presence of these contaminating liquids. Clayey sands and sandy silts are considered as soils with low to very low permeability ($k 10^{-5}$ - 10^{-8} m / s). In these soils, the circulation of water from the well leaves substances, by filtration, including organic components which transform the yellow colour of the sediments into dark tones, in the area immediately surrounding the walls, within 1-1.5 metres (see the difference between yellow and dark sediments in Fig. 1). In the case of a slightly acidic pH,

the carbonate component of the bones in this area can undergo dissolution, creating a soft and fragile consistency. These characteristics are observed in the skeletons next to the wells.

Discontinuous strata of dark soil were found in levels above the burials, interpretable as organic residue fills deposited in natural depressions shaped by the action of river flow and landfill pits. These strata were of varying depths, and some of them were almost in contact with the burials (Fig. 2). They derive from rapid and transient superficial accumulations of domestic waste consisting of solid organic material, only partially decomposed and subject to washout and evapo-transpiration. Therefore, the infiltration of these accumulations into the low permeability soil of the necropolis would appear to be negligible.

The status of each sampled individual in this study in relation to the overlying dark layers and later medieval wells is summarized in *Supp. mat. 1.*

2.2 Archaeological samples

During excavations, 22 sediment samples were collected in the pelvic area of 18 individuals. In a previous study following the RHM (Rehydration – Homogenization – Microsieve) protocol for helminth egg extraction, we detected Ascarid-type eggs under light microscopy in six of those samples, corresponding to five individuals (Roche et al., 2019).

Five of the 22 previously studied samples were then used for aDNA analyses. Three of them tested positive for Ascarid-type eggs under the microscope, while two of them did not contain any eggs. These samples were selected in order 1) to confirm our previous microscopy-based diagnosis, 2) to test for the suspected presence of other taxa, and 3) to search for the presence of parasite aDNA in samples considered to be negative up to now. Each sample originally consisted of ~100 grams of sediment collected directly in the field. For aDNA analyses, each

sample was extracted twice (2 x 5 grams of sediment) and each extraction was treated as a single sample. Therefore, we will refer to 10 samples in the following part of this paper, considering that each original field-collected sample was analysed twice.

Among the five samples originally collected in the field from five different individuals, one was collected in the lumbar vertebra area of the corresponding skeleton, one was collected on the sacrum bone, one was collected on the coccyx, and the last two were collected on the pelvis of their corresponding skeletons (Table 2).

No field control samples were available for this study.

2.3 Precautions to avoid contamination

In order to avoid cross-contamination, sediment samples were stored and subsampled in a special storage room where no molecular analyses are conducted. The subsamples intended for sediment aDNA analyses were then stored at 2-4° C and extracted in a room reserved for this process. Following extraction, products were stored at -20° C until they were transferred to a third, physically separated room reserved for PCR-based amplification. PCR products are never brought back to the preceding rooms and are stored in their own fridge at -20° C until cloning or direct sequencing. No DNA from modern parasite tissues is extracted or amplified in those rooms, and no modern positive controls were used in our experiments.

2.4 Sediment aDNA extraction

DNA extraction was conducted using DNeasy PowerMax Soil Kit (Qiagen). This kit was specifically developed for highly degraded and diluted short DNA sequences from environmental samples.

Five grams of dry sediment per sample were added to C1 and powerbead solution in bead tubes before being vortexed for mechanical and chemical cellular lysis purposes and centrifuged. Supernatant was transferred into a clean collection tube in order to start the purification process with the subsequent addition of C2 and C3 inhibitor precipitation solutions. The purification product was then centrifuged through a silica column for DNA isolation, washed, and eluted in a new collection tube.

Extraction products were then concentrated in 3M sodium acetate pH 5.5 and 70 % ethanol solutions overnight at -20°C. Nucleic acids were resuspended in 100 µl sterile water. The concentration was estimated at 260 nm absorbance on a spectrophotometer (NanoVue Plus – GE). An extraction blank was processed alongside sediment samples.

2.5 PCR amplification and sequencing

Primers were previously designed to target several gastrointestinal species (Côté et al., 2016). The primers used in the present paper are listed in Table 1. They allow us to distinguish *Ascaris* sp., *Trichuris trichiura*, *Enterobius vermicularis*, *Fasciola hepatica*, *F. gigantica*, *Dicrocoelium dentriticum* and *D. chinensis*.

In order to avoid type II errors as much as possible, which are dependent on previously recorded aDNA constraints, we planned our experiment as follows. Each of our five originally collected sediment samples was extracted twice (Fig. 3). We then obtained 10 separated samples consisting of 5 grams of dry sediment. PCR were then processed and triplicated for each primer pair, leading to 150 PCR experiments.

For PCR purposes, we used Accustart II PCR ToughMix reaction cocktail (Quantabio). Again, this kit is specifically designed to overcome commonly encountered PCR inhibitors from environmental samples. Consequently, this kit offers an applied and ready-to-use solution for

routine diagnosis in molecular paleoparasitology from layer, sewer, latrine or intestinal samples.

Two microlitres of previously obtained concentration products (5 ng / μ l) were then added to enzymatic solutions for each primer pair. Enzymatic solutions were prepared using the Master-Mix method in order to minimize cross-contamination. Each primer pair was attributed an optimized PCR program for simplex approach (Supp. mat. 2). PCR negative controls were included. PCR products were visualized by 2-3.5 % TBE-based agarose gel electrophoresis stained with SYBRgold (Invitrogen). One positive PCR product was subsequently selected per primer pair, blunted and ligated into plasmid pJET1.2/blunt Cloning Vector (Thermo scientific), transformed into *Escherichia coli* DH10B competent cells and spread onto Luria-Bertani's (LB) agar plates with ampicillin (100 μ g mL⁻¹).

Two randomly picked colonies per primer pair were screened for the presence of the correct size insert by BglIII restriction digestion (Thermo scientific) and selected recombinant plasmids were purified (GeneJET Plasmid Miniprep kit, Thermo scientific) for sequencing analysis.

DNA sequencings were performed by Biomnigene SAS (<https://www.biomnigene.fr>, Besançon, France) and nucleotide sequences were determined on both strands.

2.6 Sequence identification

Sequences were compared to the GenBank nucleotide database using the BlastN program ([http:// www.ncbi.nlm.nih.gov/Blast/](http://www.ncbi.nlm.nih.gov/Blast/)).

3. Results

Out of 150 PCR experiments conducted for template samples, 29 (19.3%) tested positive for size-based diagnosis under Bio-Rad illuminator after electrophoresis (Table 2). Asc2 primers tested positive in 18/30 PCR, while Trich4 tested positive in 10/30 experiments and Dicro22 tested positive in only 1/30 PCR reactions. Fas3 and Entero4 primers did not work at all and did not show excessive non-specific bands or primer-dimers. PCR triplicates did not systematically show 3 positive results. Indeed, while Asc2 primers were successful in 3/3 PCR experiments the majority of the time, the same is not true for Trich4 and Dicro22, which were detected in one or two PCR each time with the exception of Trich4 in Extraction 4 (3/3). Only 1/10 samples (Extraction 4-b) did not show any positive result. With the exception of Asc2, primer pairs were not successful in both extraction products for each individual. In terms of individuals, all of them (n=5) tested positive for at least one parasite. One individual tested positive for *T. trichiura* only (T17 IND B). Three individuals tested positive for *Ascaris* sp. and *T. trichiura* (T9 IND C, T18 IND B and T22 IND B), and one individual tested positive for *Ascaris* sp., *T. trichiura* and *D. dendriticum* (T8 IND C, Fig. 1). Extraction and PCR blanks remained negative.

One positive PCR product per primer pair was cloned and the obtained inserts were subsequently sequenced. All three samples were positive for their respective target, aligning with *Ascaris* sp., *Trichuris trichiura* and *Dicrocoelium dendriticum* genomes through BlastN research (Table 3). Sequence alignments and inferred phylogenies confirmed their close relationship with the targeted taxa (Fig. 4).

4. Discussion

4.1 PCR results and differential aDNA preservation

Although a minority of the PCR reactions were successful, all of our individuals tested positive for parasitism contrary to previous microscopy-based diagnosis. Except for *Ascaris* sp., positive PCR tended to be obtained in only 1/2 extraction products per individual. This is not surprising as, for each extraction, 5 grams of sediment were subsampled from a larger 100 gram volume. As aDNA is not necessarily homogeneously distributed and/or preserved in this initial volume, we expected to find positive and apparently negative subsamples in the same initial volume, as previously suggested (Côté et al., 2016). The same is true for PCR triplicates. Asc2 primer triplicates probably benefitted from the high number of eggs initially disseminated from *Ascaris* adult worms. Indeed, up to 200,000 eggs can be laid per day and per female in *Ascaris* sp., as opposed to 5,000 per day and per female for *T. trichiura* (Bogitsh et al., 2012). Moreover, *Ascaris* sp. eggs are double-shelled and do not possess mucous polar plugs, unlike *T. trichiura*. *Dicrocoelium* worms can shed a few hundred to a few thousand eggs per day per gravid individual depending on the host species (Beck et al., 2015). Its typically operculated eggs may have prevented optimal aDNA preservation, contrary to fully closed and double-shelled *Ascaris* eggs. It is reasonable to suggest that a better preservation of *Ascaris* sp. aDNA can be explained by the fact that their robust and abundant eggs allowed us to detect them through full triplicates, contrary to other parasites more subject to taphonomical constraints and DNA degradation, which prevent optimal *Taq* polymerase activity through different PCR reactions. Indeed, PCR inhibition in environmental and ancient samples is a well-known problem (Baar et al., 2011; Schrader et al., 2012). It should be underlined that Ascarid-type eggs were poorly preserved under the light microscope and had generally lost their outer shell. Meanwhile, cellular content was observed in several specimens (Roche et al., 2019). This poor state of preservation not only decreased the chances

of recovering exploitable ancient nucleic acids but may also have jeopardized their stability under stringent extraction chemicals, as was suggested for the PowerMax Soil C3 solution (Murchie et al., 2020).

The number of worms per host may vary greatly depending on the severity of infection. Meanwhile, we can hypothesize that the initial global biomass per worm taxon is probably more important in accounting for the preservation of aDNA than the initial number of individual worms. For example, *Ascaris* female worms can reach 20 to 40 cm in length for 3 to 6 mm wide, while *Trichuris* worms vary from 30 to 50 mm in length (Roberts and Janovy, 2012) and *Dicrocoelium* worms vary from 8 to 14 mm in length for 2 to 3 mm wide (Otranto and Traversa, 2002). As aDNA molecules are known to be mostly preserved in extracellular forms bound to environmental minerals and humic acids (Rawlence et al., 2012), not only the initial higher number of eggs and their intrinsic preservation characteristics, but also the initially available tissue biomass from adult worms, may have allowed for a better preservation and recovery of extracellular *Ascaris* sp. DNA. This well-known phenomenon in aDNA studies also explains the detection of parasite DNA, even in the absence of observable eggs or their cellular content.

4.2 Parasite localization throughout the intestinal tract and DNA leaching

The parasites detected in this study were not systematically observed among the five tested individuals. Only one individual tested positive for *D. dendriticum* (T8 IND C) while *Ascaris* sp. was barely detected in one individual (T18 IND B), and not at all in another (T17 IND B). Similarly, *T. trichiura* was barely detected in one individual (T9 IND C) with only one positive PCR reaction, while another (T17 IND B) showed three positive PCR reactions (Table 2).

Interestingly, these results may be related to rough sample anatomical location. Indeed, intestinal parasites generally occupy separate and well-identified ecological niches inside their host, depending on the species. Considering gastrointestinal parasites, those niches consist in different regions of the host digestive tract where sexually mature worms meet and reproduce. While the complete developmental cycle of those worms may involve extended areas outside the strict digestive system (e.g., *Ascaris* sp. juveniles migrating through the respiratory system, see Bogitsh et al., 2012), the bulk of their biomass is to be found in the region where adult worms finally settle and produce thousands of new eggs. This explains why *Ascaris* sp. tended not to be detected in the two samples collected on pelvis bones in our record. This may be related to the biological cycle of this worm living in the host small intestine in its adult stage (Bogitsh et al., 2012). The mostly central localization of the small intestine may have favoured parasite detection in samples closer to the central body axis, namely the lumbar region, the sacrum bone and the coccyx. Conversely, more remote samples, such as those collected on the pelvis bone do not appear to be conducive to detection. It should be underlined that Ascarid eggs were observed in two pelvis bone samples in a previous study (Roche et al., 2019), but were not detected in this type of sample most of the time. The same reasoning may be applied to *T. trichiura*, which was detected in all types of sample locations, with the most positive PCR from a pelvis sample. This may account for the normal colonization of the caecum and ascending colon by *T. trichiura* adult worms (Bogitsh et al., 2012), directly overlying the pelvis bone. The detection of *T. trichiura* farther in the colon is easily explained by the migration of eggs toward the host's descending colon and anus. The same is true for *Ascaris* sp. eggs. Therefore, it is not surprising to find both of them quite high in the vertebral region (T8 IND C), in close proximity with the transverse colon, where newly released eggs migrated, but also in the duodenum region where newly ingested eggs descended towards their hatching zone in the small intestine. Similarly, the discovery of *D. dendriticum*

DNA in the same region may account for the migration of eggs in the transverse colon or duodenum on one hand, or for its close proximity with bile ducts on the other hand, where adult worms are normally found (Roberts and Janovy, 2008) and therefore the bulk of their initial biomass. This last point only stands in case of a true infection by *D. dendriticum*. In this regard, it is noteworthy that *D. dendriticum* was only detected in the sample collected from the lumbar area.

Nonetheless, it is important to recall that this individual positive to *D. dendriticum* was the only one with a well cutting through its lower part (Table 1 and 2; Fig. 1). Pollution from the well contents can be suggested to explain the detection of *D. dendriticum* in this individual and is corroborated by taphonomical observations.

DNA leaching is still an open-ended matter in aDNA studies with several papers clearly demonstrating its occurrence in ancient and modern contexts (Haile et al., 2007; Andersen et al., 2012), particularly in non-discrete samples from open and non-frozen landscapes (Rawlence et al., 2014). Conversely, studies on DNA leaching do not show its systematic occurrence, even in wet and regularly flooded river bank environments (Hebsgaard et al., 2009; Andersen et al., 2012).

This topic can be examined by further analyses at the Uffizi Gallery burial site, namely through the systematic sampling of the overlying strata and relative DNA quantification from medieval dark earth through underlying alluvial deposits and Late Antique funerary pits. Still, although large mammal DNA may be retrieved several tens of centimetres under the original soil (Andersen et al., 2012), the leaching parasitic gastrointestinal fauna DNA would be negligible compared to the biomass of worm tissues directly available in the decaying digestive tract of their host. This assumption may be better substantiated through the

controlled approximation of vertical and horizontal DNA leaching from the overlying dark layers and around dispersion wells. In this way, the same methods may be applied to map relative parasite DNA availability throughout the host body cavity by a systematic quadrats sampling strategy scaled to single or multiple individual graves and DNA quantification. This may enable us to test the reliability of the previously exposed assumption regarding parasite distribution across our record depending on anatomical sample location. Obviously, exogenous bioturbation but also endogenous body decay processes and various funerary contexts and practices (shrouds, coffins...) may interfere with initial parasite distribution inside and outside their initial host body volume. In the case of the Uffizi Gallery burial site, the absence of coffins certainly limited the presence of empty spaces around decaying bodies and therefore prevented the circulation of body fluids after death. Similarly, the use of this funerary area for a seemingly single mass death event (possibly spanning several days or weeks) prevented soil reworking associated with the mixing of sediments from different individuals, which is observed in cemetery areas used over long periods of time.

4.3 Detected parasites, their frequency and comparison with microscopy-based diagnosis

Previous microscopic observation allowed us to select one candidate individual (T9 IND C) for targeted aDNA detection based on the number of Ascarid-type eggs per six slides in this individual. Ascariasis seems to be well established in this population subgroup (4/5 individuals) as aDNA fragments confirmed the previous observation of Ascarid-type eggs in three of them and added one positive individual that was not tested positive under the microscope. Successful and confirming results using Asc2 primers highlighted the potential yields of an aDNA approach at this site. As previously suggested, *T. trichiura* was finally detected in 5/5 individuals through aDNA, while totally absent under microscopy (Roche et

al., 2019). Indeed, the biological cycle and environmental conditions necessary for the dissemination of *T. trichiura* are very similar to *A. lumbricoides* and consequently, those two nematodes are commonly found infesting a same individual (Howard et al., 2001). In the same way as for *A. lumbricoides*, the presence of *T. trichiura* in 5/5 individuals is clearly consistent with the ubiquitous nature and the direct life cycle of those two nematodes. On the other hand, dicrocoeliasis is a very rare infection in humans and its detection is generally a sign of false parasitism (Magi et al., 2009). Consequently, its rarity in this subgroup is also in keeping with its currently known biology and epidemiology.

4.4 Causes and consequences of the observed parasitic burden

The parasitic burden observed in those five individuals is mainly related to poor sanitary conditions. *Ascaris* sp., most probably *A. lumbricoides* in this anthropic context, and *T. trichiura* are faecal-oral transmitted parasites. Contamination generally occurs through spoiled hand-to-mouth contact, ingestion of contaminated drinking water, or vegetables polluted by human excreta. The use of human crop fertilizers, the absence of modern flushing toilets and overcrowded communities dramatically increase the risk of infection. Ascariasis and trichuriasis are globally asymptomatic nowadays in the Western World and their prevalence is very low in general in this area. In less advantaged regions however, these otherwise mild infections can lead to nutritional spoliation, growth impairment, abdominal pain, diarrhoea, intestinal occlusion or anaemia (Crompton, 2001; Savioli and Albonico, 2004; de Silva and Cooper, 2011). In a context of food shortages and an ineffective public health system, they can dramatically accentuate the impact of famine and viral or bacterial co-infection. Those conditions were almost certainly encountered on a regular basis among ancient populations in Late Antique Italy (Mitchell, 2017). The situation of decline at that

time in ancient Florence, the destruction of the city aqueduct, and a probable epidemic of still unknown aetiology were the perfect grounds for an increased impact and spread of those parasites, as was previously suggested (Roche et al., 2019). Our new results confirm our previous hypotheses in this regard concerning a wider frequency of parasitic infections in this population, and co-infection with closely related species. Meanwhile, it is important to recall that ascariasis and trichuriasis are well known in the Western World since Prehistory (Le Bailly et al., 2012), and become increasingly prevalent during Antiquity (Le Bailly et al., 2011; Dufour, 2015). Thus, a reliable assessment of the impact of changing environments and cultural stressors (e.g., wartime) on the frequency and burden of parasitism in past populations remains an upcoming challenge for paleoparasitologists in the near future.

D. dendriticum is not related to poor sanitary conditions but to food habits. Indeed, this liver fluke commonly infects cows and sheep. Its eggs are released in the environment where larvae are successively eaten by terrestrial snails and ants. The behaviour of infected ants is modified so that they climb onto the top of blades of grass where they are accidentally ingested by grazing ruminants. The infective larvae then migrate from the ruminant's gut to the liver, where adult worms produce new eggs (Ortando and Traversa, 2002). Consequently, humans rarely enter the biological cycle of *D. dendriticum*, and the discovery of its eggs in human faeces is often the sign of false parasitism. This false parasitism is caused by the consumption of infected sheep or cow liver. The ingested eggs are passed through the digestive tract without causing a true infection.

Dicrocoelium sp. eggs were previously detected in the paleoparasitological record in Europe during the Roman period (Le Bailly and Bouchet, 2010). In Italy, they were retrieved from a sediment core in Roman Ostia, and from medieval pits in Parma (Florenzano et al., 2012; Dufour, 2015). In such contexts, it was not possible to link the eggs to false parasitism or true infection as they were observed outside body remains and may simply have been laid by

ruminant hosts. On both occasions, they were observed in conjunction with human and non-human animal infecting parasites.

The consumption of cattle and sheep meat is well known in Roman times, as shown by the archaeozoological record, particularly in Italy (MacKinnon, M.R., 1999). Written data on culinary habits contribute precious information from the period of the Uffizi Gallery emergency burial site (Mackinnon, M., 1999; Moses, V., 2012). From the beginning of the 4th c. CE, the Edict of Diocletian (*Edictum de pretiis rerum venalium*) aimed to obstruct price increases for numerous goods, such as food, by setting maximum prices. The price for sheep meat, among others, was thus set, but there is no mention of specific organs, contrary to the *Ficatium* or specially enlarged hog's liver by fattening. But from the end of the 4th c. CE, the Apicius' Cookbook (*De re coquinaria*) details numerous recipes and ingredients. Liver from different animals enters many of these recipes, including cooked sheep's liver.

It is difficult to assess the true popularity of such culinary delicacies at that time, and sheep's liver may have been restricted to the diet of the Roman elite, to a certain extent. Meanwhile, it is reasonable to assume that appreciated culinary habits in past populations led to different patterns in terms of false or true parasitism, as they exposed consumers to foodborne infections differently (Reinhard et al., 2013). Stable isotope analysis coupled with paleoparasitological diagnosis may allow for a better estimation of the relative importance of false or true foodborne diseases by revealing the staple diet of past populations. However, it is important to recall that true *dicrocoeliasis* can be observed in humans (Magi et al., 2009). The observation of *D. dendriticum* eggs in the liver of an infected human is the only way to prove a true infection in the past, rather than a situation of false parasitism caused by the consumption of infected organs. Such an observation was recorded in the liver tissue of the Zweeloo Woman bog mummy dated to the Roman period in the Netherlands (Searcey et al., 2013). But if *dicrocoeliasis* did indeed occur in the past, as demonstrated by the Zweeloo

Woman case, individual detections such as in the Uffizi Gallery burial site do not allow for a definitive diagnosis of true or false parasitism. More paleoparasitological studies in funerary contexts in societies where liver consumption was diversely appreciated will certainly lead to substantiated tendencies in the future.

Moreover, the comparison of the detected parasitic diversity with paleodemographic data including sex, age at death, and pathological conditions, might allow for a proper paleoepidemiological assessment of parasitic infection dynamics in the ancient population of the Uffizi Gallery emergency burial site.

5. Conclusion

Our combined paleoparasitological approach was guided by an exploratory microscopic diagnosis, which is currently the most widely used type of analysis. This allowed us to identify egg-containing samples to test for aDNA extraction and comparison. Our targeted approach led us to detect the presence of *Ascaris* sp., *Trichuris trichiura*, and *Dicrocoelium dendriticum* aDNA fragments. Although only one taxon (Ascarid-type eggs) was detected under the microscope in three individuals among five later tested for aDNA, all of those five individuals tested positive for at least one parasite after aDNA extraction. *Ascaris* sp. was detected in the three previously identified individuals as containing Ascarid-type eggs, but also in one supplementary individual where no eggs were previously detected. Moreover, two more taxa were detected through aDNA extraction, including *T. trichiura* detected in all five individuals. Four individuals tested positive for both *Ascaris* sp. and *T. trichiura*, while one individual tested positive for the latter only. This suggests a very widespread parasitic burden related to the faecal pollution of soils, drinking water and food in ancient Florence at a

difficult time, including co-infection by possibly debilitating nematodes. This burden may have played a role in aggravating a possible epidemic or other mortality crises that plagued this community when this emergency burial site was set up. *D. dendriticum* was detected in one individual who also tested positive for *Ascaris* sp. and *T. trichiura*. The consumption of ruminant liver is well known in Roman times and may have led to higher proportions of false parasitism than in other populations. But true infection by *D. dendriticum* is also known from the Roman period and it is not possible to choose between one or another hypothesis at this individual scale. Moreover, the possibility of a false infection by *D. dendriticum* should not be taken for granted considering the risk of DNA leaching from late medieval layers in this particular case.

More paleoparasitology studies combining molecular and microscopic approaches conducted in funerary contexts will allow us to better distinguish reliable approximations of past infection dynamics in populations through time from potentially confounding factors, such as DNA leaching from overlying strata, or fluctuating DNA preservation and availability depending on the anatomical localization of samples in the host abdominal cavity.

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Fig. 1 T8 under excavation, showing several individuals in a same burial. White arrow: indicates Individual C. This individual was cut by a late medieval well in its inferior left side in the pelvis area (see Table 1). One sediment sample was collected in the undisturbed lumbar area.

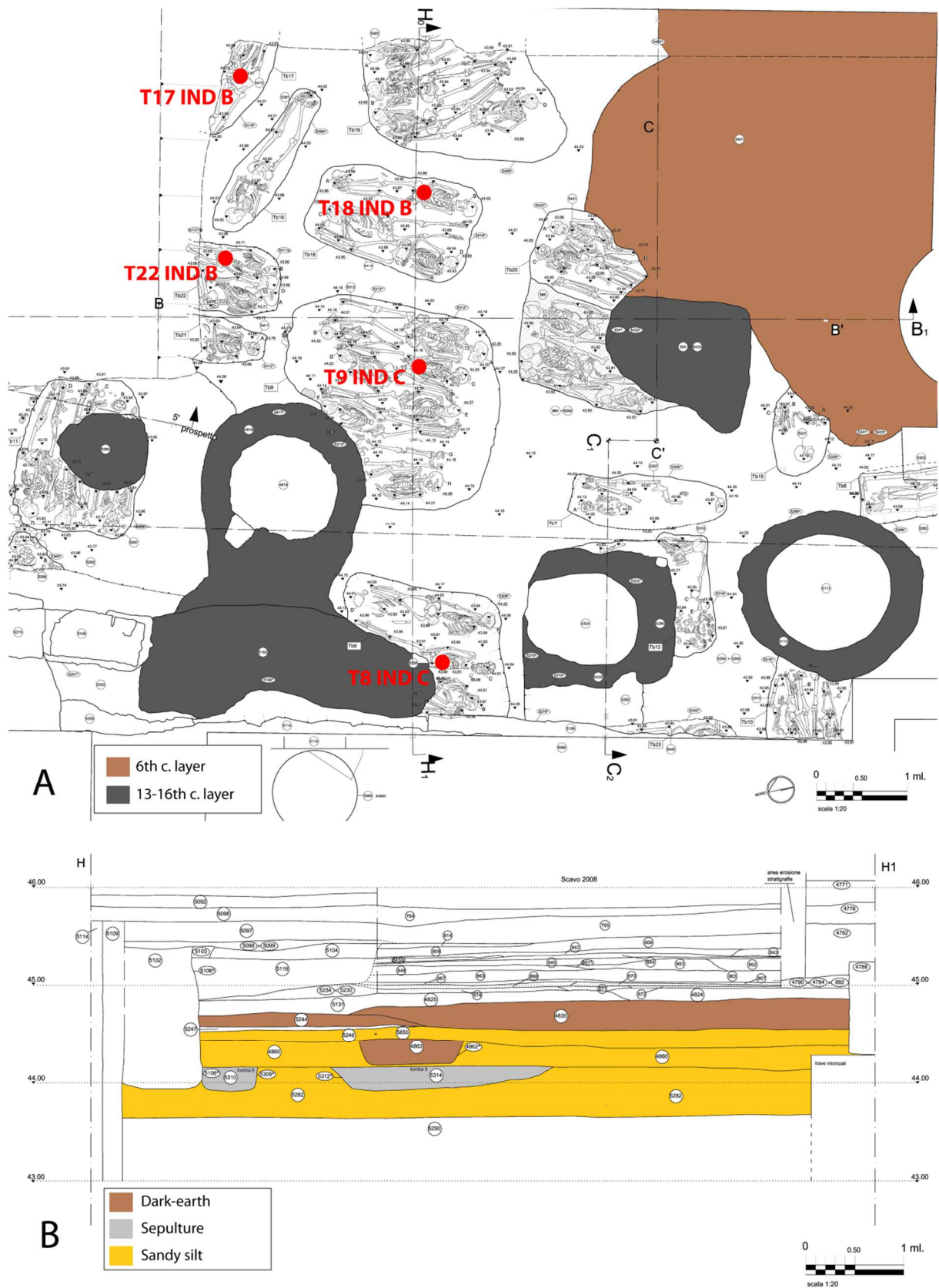


Fig. 2 A) Location of the sampled individuals (red) in the burial area in regard of later perturbations. B) Stratigraphic view showing the intercalation of dark-earth layers, sandy silt deposits, and sepultures containing T8 IND C (left) and T9 IND C (right).

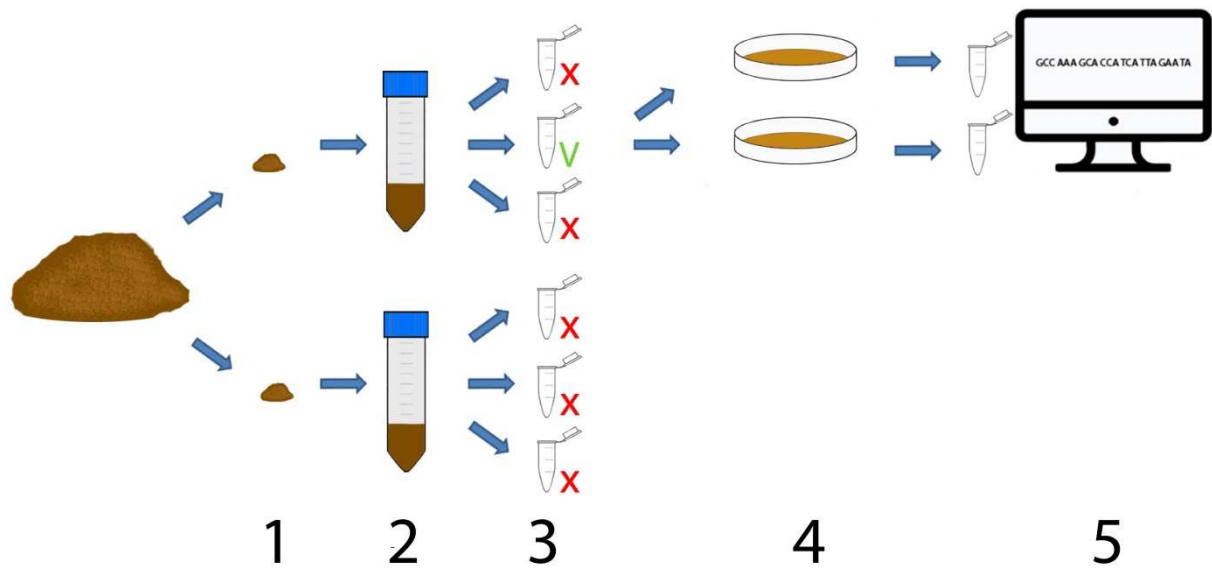


Fig. 3 Summarized experiment design. 1) One original sample from one individual is subsampled twice. Each subsample equals 5 grams of dry sediment. 2) Each subsample is extracted through bead-beating and silica-based whole DNA sequestration. 3) Each PCR-based amplification is triplicated per primer pair. 4) Positive PCR products are cloned. 5) Grown clones are extracted and sequenced.

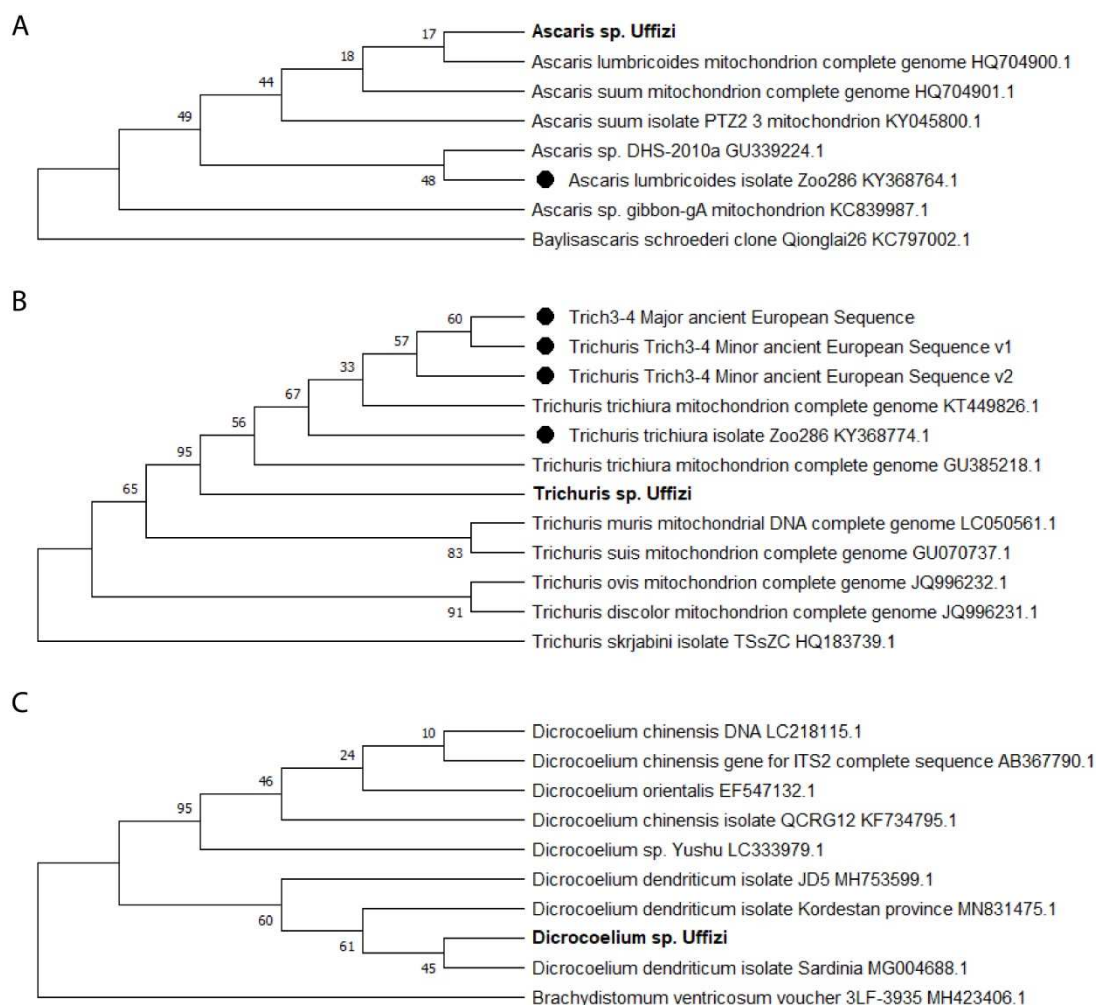


Fig. 4 Phylogenetic interrelationships of isolated sequences (bold) in regard of ancient (dots) and current strains of their respective species. Trees were inferred through Maximum likelihood (ML) with 1000 bootstraps after alignment in MEGA X software. All bootstrap values are shown. A) As expected, the *Ascaris* fragment is indistinguishable from *A. lumbricoides* and *A. suum* complete genomes (respective GenBank accession numbers HQ704900.1 and HQ704901.1) as it showed 100% homology with both sequences. Thence the unreliable bootstrap values for proposed bifurcations. *B. schroederi* was used as an out-group. B) The obtained *Trichuris* fragment clearly falls within *T. trichiura* diversity, currently infesting primates. Ancient Trich3-4 sequences were from Côté et al., 2016. *T. skrjabini* was used as an out-group. C) The obtained *Dicrocoelium* fragment groups within *D. dendriticum* diversity and is separated from *D. chinensis*. *B. ventricosum* was used as an out-group.

Primer pairs	Targeted species	Targeted genes	Size of amplicons (bp)
Asc2	<i>Ascaris lumbricoides</i> <i>Ascaris suum</i> *	Cytochrome b (cytb)	74
Trich4	<i>Trichuris trichiura</i>	Large ribosomal subunit (LSU)	91
Fas3	<i>Fasciola hepatica</i> <i>Fasciola gigantica</i>	rRNA18s (nuclear)	85
Dicro22	<i>Dicrocoelium dendriticum</i> <i>Dicrocoelium chinensis</i>	Internal transcribed spacer 2 (nuclear)	76
Entero4	<i>Enterobius vermicularis</i>	Cytochrome oxydase 1 (cox1)	53

* Asc2 does not discriminate *A. lumbricoides* from *A. suum* contrary to Fas3 with *Fasciola* species.

Table 1 Primer pairs used in this targeted approach, previously designed in Côté et al., 2016.

Individuals	Sample localization	Extractions	Nbr of positive PCR reactions per primer pair					Ascarid type eggs	Disturbances
			Asc2	Trich4	Dicro22	Fas3	Entero4		
T9 IND C	Sacrum	Extrac_1	3	1	0	0	0	Yes	Cut pit
		Extrac_1-b	3	0	0	0	0		
T8 IND C	Lumbar vertebrae	Extrac_2	3	0	1	0	0	Yes	Cut ind.
		Extrac_2-b	3	2	0	0	0		
T22 IND B	Coccyx	Extrac_3	2	2	0	0	0	Yes	Not cut
		Extrac_3-b	3	0	0	0	0		
T17 IND B	Pelvis	Extrac_4	0	3	0	0	0	-	Not cut
		Extrac_4-b	0	0	0	0	0		
T18 IND B	Pelvis	Extrac_5	0	2	0	0	0	-	Not cut
		Extrac_5-b	1	0	0	0	0		

Table 2 Results for 150 PCR experiments. Each primer pair is tested 3 times per extraction product. This aims to minimize type II errors due to aDNA degradation and scarcity, and amplification fails from environmental inhibitors. PCR results are shown against microscopy results (Ascarid type eggs). Disturbances refer to the presence of later wells cutting the pits (Cut pit) or the individual (Cut ind.) contained in it.

Species	Targeted gene	Obtained sequence	Size (bp)	Homology	E-value	Origin
<i>Ascaris</i> sp.	Cytochrome b (Cyt b)	GGTATGGTTTTGGGTTTTTCAGATTTTGACTGGTACTT TTTTGGCTTTTTATTATTCTAATGATGGTGCTTTGGC	74	100%	3e-28	Clones
<i>T. trichiura</i>	Large ribosomal subunit (LSU)	GGTTTAAACTCAAATCACGTAATGTTTATCGTCGAAC AGACGAGAATAAAAATTCTTCTGCAAACATATATAC AAATGATTCAACATCGAA	91	97,78%	5e-34	Clones
<i>D. dendriticum</i>	Internal transcribed spacer 2 (ITS2)	TACACACACCTAGTTATCAGACAGGTGGAGATGTGT CTACGGAGTCGTGGCTCAGTAATATTTATGCGCGGTC TGT	76	98,68%	1e-27	Clones

Table 3 Obtained sequences with their respective percentage of homology and E-value for the expected target species through BlastN research.