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Amanda Rollins

Krystiana Krupa

Georgia Millward

Dario Piombino-Mascali

Karl Reinhard

See next page for additional authors

Authors

Amanda Rollins, Krystiana Krupa, Georgia Millward, Dario Piombino-Mascali, Karl Reinhard, and Frederika Kaestle

Molecular identification of parasites in an intestinal coprolite from a mummified religious dignitary of the Piraino Mother Church crypt, Sicily

Amanda Rollins,[1] Krystiana Krupa,[2]
Georgia Millward,[2] Dario Piombino-Mascali,[3]
Karl Reinhard,[4] and Frederika Kaestle [2]

1 Department of Biology, Wilmington College, Wilmington, USA
2 Department of Anthropology, Indiana University, Bloomington, USA
3 Department of Anatomy, Histology, and Anthropology, Vilnius University,
    Vilnius, Lithuania
4 School of Natural Resources, University of Nebraska-Lincoln, Lincoln, USA

Corresponding author — A. Rollins, Wilmington College, 1870 Quaker Way,
    Wilmington, OH 45177, USA. *email* amanda_rollins@wilmington.edu

Abstract

Intestinal contents were sampled from a spontaneously enhanced mummy from the Sepulcher of the Priests of the Piraino Mother Church in the Province of Messina, Sicily. This adult male mummy, Piraino 1, is an unidentified religious dignitary dating from the late-18th to mid-19th centuries. Immunological and molecular diagnostics were used to test for common and clinically significant parasites. A morphological diagnosis of *Trichuris trichiura* (whipworm) was confirmed genetically. A previously undetected *Enterobius vermicularis* (pinworm) infection was

also identified genetically. These data indicate that the Piraino 1 individual was simultaneously infected with multiple intestinal parasite species indicative of poor hygiene. This study also emphasizes the importance of utilizing multiple diagnostic techniques to detect pathogens from archaeological contexts.

Keywords: Archaeoparasitology, Paleoparasitology, Piraino, Mummy, Paleogenetics, Ancient DNA, Helminth, PCR, *Trichuris trichiura, Enterobius vermicularis*

1. Introduction

The Sepulcher of the Priests is a crypt located in the Mother Church of the town of Piraino in the Province of Messina, Italy. This three-chambered crypt contains the mummified remains of several male clergymen, clothed and displayed in horizontal and vertical niches (Piombino-Mascali et al., 2017). Archival sources are available for 23 of these 26 clergymen, indicating that they were interred between 1773 and 1858. These mummies were spontaneously enhanced, meaning that the morticians took advantage of known natural mummification conditions to preserve the deceased (Aufderheide, 2003; Cardin, 2014). Cadavers were drained by being placed upon a wooden rack to dehydrate and mummify (Fornaciari et al., 2010). Such preservation of socially distinguished and religious dignitaries was a common practice in Sicily and Southern Italy at the time (Piombino-Mascali et al., 2017; Piombino-Mascali and Nystrom, 2020).

Within the framework of the Sicily Mummy Project (2007–2021), a physical examination of one of these mummified clergymen, Piraino 1, revealed dental calculus, enamel hypoplasia, and pleural adhesions; radiographs revealed degenerative changes of the spine as well as numerous osteolytic changes in the skull, ribs, humerus, and os coxae (Piombino-Mascali et al., 2013, 2017). Visceral samples collected from a minimal opening in the abdomen of Piraino 1 included some intestinal contents, or coprolites. Care was taken to avoid contamination, and the specimens were sealed in a sterile plastic container prior to analyses. Microscopic archaeoparasitological analysis revealed the heaviest recorded infection of whipworm, *Trichuris trichiura*, despite suboptimal morphological preservation of the eggs (Kumm et al., 2010). An infection of this parasitic helminth usually results from the consumption of soil-contaminated food in areas of poor hygiene and environmental fecal contamination (Garcia, 2007).

Botanical analyses revealed a predominance of *Polygala vulgaris* pollen, signifying that the Piraino 1 clergyman had consumed a tea made with the flowers of this plant, which was historically used for medicinal purposes (Piombino-Mascali et al., 2013; Nystrom and Piombino-Mascali, 2017).

Certainly, the Piraino 1 individual suffered from multiple illnesses and was likely being medicated near the time of his death. Unfortunately, not all paleopathological conditions can be identified morphologically. In order to gain a comprehensive view of comorbidity of diseases in the past, immunological and genetic analysis can be included to confirm morphological diagnosis, as well as to test for other common infectious agents that might be degraded beyond recognition or present in too few numbers to be detected. It was expected that the application of genetic and immunological methods to the Piraino 1 coprolite would reveal additional evidence of parasitological infections. We focused our testing on parasites of clinical importance, particularly those that do not preserve well.

2. Methods

DNA extractions and immunoassays were performed in the Ancient DNA Laboratory of Molecular Anthropology housed at the Indiana Molecular Biology Institute at Indiana University. This laboratory is used only for ancient DNA extraction, and it is under positive-pressure, HEPA-filtered, and UV-irradiated. Standard ancient DNA laboratory protocols were followed (Kaestle and Horsburgh, 2002; Pääbo et al., 2004). Coprolite samples were irradiated externally prior to rehydration. Reconstituted coprolite solutions were then subjected to immunological and molecular testing.

For the immunological tests, 150 mg samples of central coprolite sediment were rehydrated in 500 µl TE buffer with occasional vortexing. Enzyme immunoassays were used to detect antigens of common waterborne pathogens, *Giardia* and *Cryptosporidium*, using the TECHLAB ® *GIARDIA/CRYPTOSPORIDIUM QUIK CHEK*™ (Alere) kit, following kit protocol for diluted samples. The coprolite solution was also tested with the TECHLAB® *E. HISTOLYTICA QUIK CHEK*™ (Alere) kit, which can differentiate the presence of *Entamoeba histolytica*

antigens from those of the related non-pathogenic *Entamoeba dispar*. Both positive and negative controls were included for each kit.

To conduct the molecular analyses, 150 mg sediments from the center of irradiated coprolites were added to 400 μl digestion buffer (100 mM NaCl, 50 mM Tris, 50 mM EDTA, 1% SDS), along with 20 μl 1 M DTT, 60 μl 10 mg/ml proteinase K, and 100 μl 10% SDS, and incubated at 56 °C for 72 h, following Iñiguez et al. (2003). Digested sample solution was then extracted with the Isolate Fecal kit (Bioline No. BIO-52037) according to the kit protocol. Negative controls were included in every extraction and amplification.

Ancient DNA analyses included molecular primers designed to specifically test for *Trichuris trichiura*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *Entamoeba histolytica*, *Giardia duodenalis*, *Necator americanus*, *Ancylostoma duodenale*, *Cryptosporidium parvum*, and *Mycobacterium tuberculosis* (**Table 1**). Because *T. trichiura* had been previously identified through archaeoparasitological microscopy of this coprolite (Kumm et al., 2010), primer sets specific to the 18S ribosomal RNA of this organism were used to confirm diagnosis and assess the genetic preservation of the samples (Oh et al., 2010). *A. lumbricoides* primers targeted a segment of the mitochondria cytochrome *b* gene (Loreille et al., 2001). Novel *E. vermicularis* primer sets utilized a hemi-nested reaction to improve amplification success of the cytochrome oxidase 1 (*cox1*) gene. *E. histolytica*

Table 1 Pathogen primer sets for PCR with expected sequence length. This includes internal primer sets for nested PCR reactions.

Pathogen	Primer type	Forward	Reverse	Sequence length
Trichuris trichiura	External	TCTCCATGAGACGCGTTACC	GAACGACTCCTGCTTAGGAC	189 bp
Ascaris lumbricoides	External	GTTAGGTTACCGTCTAGTAAGG	CACTCAAAAAGGCCAAAGCACC	142 bp
Enterobius vermicularis	External	GGAGGTGTTTGGTCATTTGGG	TTTACCCCAGTTGGCACAGC	169 bp
Enterobius vermicularis	Internal	AGGTGTTTGGTCATTTGGGT	TTTACCCCAGTTGGCACAGC	126 bp
Entamoeba histolytica	External	GTACAAAATGGCCAATTCATTCAATG	ACTACCAACTGATTGATAGATCAG	135 bp
Giardia duodenalis	External	CATCCGGTCGATCCTGCC	AGTCGAACCCTGATTCTCCGCCAGG	268 bp
Giardia duodenalis	Internal	GACGCTCTCCCCAAGGAC	CTGCGTCACGCTGCTC	200 bp
Ancylostoma duodenale and *Necator americanus*	External	ACGTCTGGTTCAGGGTTGTT	TTAGTTTCTTTTCCTCCGCT	310 bp
Ancylostoma duodenale	Internal	CGACTTTAGAACGTTTCGGC	TTAGTTTCTTTTCCTCCGCT	130 bp
Necator americanus	Internal	ATGTGCACGTTATTCACT	TTAGTTTCTTTTCCTCCGCT	250 bp
Cryptosporidium parvum	External	GCGAAACGATTTGCCAAGGA	AGTTTCAGCCTTGCGACCAT	195 bp
Cryptosporidium parvum	External	GGAGCCTGCGGCTTAATTTG	CCACCAACTAAGAACGGCCA	127 bp
Mycobacterium tuberculosis	External	CCTGCGAGCGTAGGCGTCGG	CTCGTCCAGCGCCGCTTCGG	123 bp
Mycobacterium tuberculosis	Internal	TTCGGACCACCAGCACCTAA	TCGGTGACAAAGGCCACGTA	93 bp

primers targeted a region of the SSU rRNA that distinguishes it from nonpathogenic *E. dispar* (Gonin and Trudel, 2003). Nested primers targeted a region of SSU rRNA unique to *G. duodenalis* (Hopkins et al., 1997; Read et al., 2002; Mahdy et al., 2009). Seminested hookworm primers were designed to differentiate between the two species at the internal transcribed region 2 of rDNA (Gasser et al., 1993; De Gruijter et al., 2005). Novel *C. parvum* primers were designed to target a short segment of the 18S ribosomal RNA gene, commonly used in molecular diagnosis of this parasite (Cunha et al. 2019). The nested *M. tuberculosis* primers target the IS*6110* insertion sequence unique to pathogens of the *M. tuberculosis* complex (Braun et al., 1998; Raff et al., 2006).

Sanger sequencing methods were used for DNA amplification. Each 25 µl reaction contained molecular grade water (Eppendorf), 1 × Master Amp PCR Enhancer (Epic Technologies), 1 × PCR Buffer (Life Technologies), 1.5 mM magnesium chloride (Life Technologies), 200 µM dNTP Mix with dUTP (Applied Biosystems), 1 mg/ml UltraPure BSA (Applied Biosystems), 0.5 µM of each primer, 2 U Platinum Taq (Life Technologies), 0.25 U AmpEraseTM Uracil N-Glycosylase (UNG) (Applied Biosystems), and 3 µl DNA template. Alternatively, Uracil-DNA Glycosylase (UDG) (New England Biolabs) was substituted in place of AmpEraseTM in some reactions. Sequencing reactions were initiated in the Ancient DNA Laboratory but amplified in a separate modern DNA laboratory using a programmable thermocycler (BioRad). Following an initial cycle at 95 °C for 5 min, reactions proceeded with 40 cycles of 94 °C for 1 min, annealing temperatures of 54-60 °C for 30 s, and 72 °C for 30 s, followed by a final extension of 72 °C for 7 min and subsequent incubation at 4 °C. For samples that replaced UNG with UDG, an initial incubation of 37 °C for 10 min, followed by an incubation of 95 °C for 10 min, was required to deactivate the UDG prior to the normal PCR incubations.

Products were visualized on 2% polyacrylamide gels stained with ethidium bromide. Bands were cut from the gels and purified with the QIAEX II Gel Extraction Kit (QIAGEN) as per kit protocol, sequenced using Applied Biosystems BigDye® protocols, and BLAST searched against all sequence data within the GenBank® database.

3. Results

The morphological diagnosis of *T. trichiura* infection (Kumm et al., 2010) was confirmed with molecular data. This was indicative of the preservation of parasite DNA within the coprolite sample. Our PCR product was 100% matched to 18 *T. trichiura* small subunit ribosomal RNA gene entries in GenBank® (**Fig. 1**). A co-infection of *E. vermicularis* was also molecularly identified. The Piraino 1 mummy sequence was > 99% matched with 32 sequences of the *E. vermicularis* partial *cox1* gene sequences included in the database (**Fig. 2**). No other significant genetic matches occurred, supporting the specificity of the primers used to detect the presence of these two parasites.

No amplicons were obtained with any of the *A. lumbricoides*, *E. histolytica*, *G. duodenalis*, *N. americanus, A. duodenale, C. parvum*, or *M. tuberculosis* primer sets. The immunoassay kits also failed to provide positive evidence for *E. histolytica*, *G. duodenalis*, or *C. parvum* infections.

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Query   1    CGAGCGGTTTAACCGCAGCAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTG   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   484  CGAGCGGTTTAACCGCAGCAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTG   425

Query   61   CTGGCACCAGACTTGCCCTCCAATAGATTTAGCCGAACTTATGTACGGCTCGATCGTTCC   120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   424  CTGGCACCAGACTTGCCCTCCAATAGATTTAGCCGAACTTATGTACGGCTCGATCGTTCC   365

Query   121  GGTAACGCGTCTCATGGAG   139
             |||||||||||||||||||
Sbjct   364  GGTAACGCGTCTCATGGAG   346
```

Fig. 2. Example of GenBank® BLAST search of Piraino 1 sequences (Query) obtained from *Enterobius vermicularis* PCR reactions. The Subject in this case is Sequence ID: MN614092.1, *E. vermicularis* isolate MA253 cytochrome oxidase subunit 1 (*cox1*) gene (Nucleotide, 2011).

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Query   1    TATTTCTATTGGTTTAATTGGTAGTGTAGTATGGGGTCATCATATGTTTACTATTGGTTT   60
             ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct   36   TATTTCTATTGGTTTAATTGGTAGGGTAGTATGGGGTCATCATATGTTTACTATTGGTTT   95

Query   61   TGATATAAGAACACGTTTGTATTTTATGGTTGCTACTATAATTATTGCTGTGCCAACTGG   120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   96   TGATATAAGAACACGTTTGTATTTTATGGTTGCTACTATAATTATTGCTGTGCCAACTGG   155

Query   121  GGTAAA   126
             ||||||
Sbjct   156  GGTAAA   161
```

Fig. 1. Example of GenBank® BLAST search of Piraino 1 sequences (Query) obtained from *Trichuris trichiura* PCR reactions. The Subject in this case is Sequence ID: JF690953.1, *T. trichiura* isolate T1 18S ribosomal RNA gene (Nucleotide, 2019)

4. Discussion

The field of paleopathology requires multidisciplinary scientific rigor and differential diagnoses (Klepinger, 1983; Buikstra et al., 2017). The utilization of multiple diagnostic methods when possible is critical for both the confirmation of a suspected disease as well as the holistic understanding of disease burden in an individual from the past. Mummy studies in particular have traditionally employed the use of a variety of diagnostic techniques (Wilcox, 2002; Zimmerman, 2011).

Our study applies multiple methods of parasite diagnosis to a mummy known to have suffered from numerous health conditions (Kumm et al., 2010; Piombino-Mascali et al., 2013, 2017). Piraino 1 was previously diagnosed with dental enamel hypoplasia, suggestive of biological stress that occurred during childhood, and pleural adhesions in his chest that suggest a prior bout of pneumonia (Piombino-Mascali et al., 2013). Piraino 1 also had numerous osteolytic lesions suggestive of multiple myeloma, or skeletal metastasis, that likely led to his death (Piombino-Mascali et al., 2013, 2017). Furthermore, this individual had the highest level of whipworm infection ever identified from an archaeological sample at that time, with over 34,000 *T. trichiura* eggs per gram of coprolite (Kumm et al., 2010). Here, by including molecular diagnostics, we add a pinworm infection, *E. vermicularis*, to his list of ailments.

Although morphological evidence of *T. trichiura* had already been identified in Piraino 1 coprolite samples, the morphological preservation of these parasite eggs was of predominantly moderate condition, with the majority being deformed and empty (Kumm et al., 2010). Many eggs were folded or fractured, and the characteristic polar plugs were typically missing. In cases such as this, in which infection levels are staggering, there are plenty of eggs available to achieve confidence of the diagnosis. However, it is often the case that relatively few parasitic eggs are present in a sample, complicating diagnoses. Ancient DNA analyses have been used to successfully identify parasites that were degraded or too scarce to be detected with traditional microscopy methods (Gųhl et al., 1999; Iñiguez et al., 2002; Cleeland et al., 2013). Here, we confirm the *T. trichiura* infection with molecular diagnostics.

Typically, *A. lumbricoides* (giant roundworm) co-infections occur with *T. trichiura*, but this parasite was not seen in morphological examinations of the Piraino 1 coprolite (Garcia, 2007; Kumm et al., 2010; Leles et al., 2010). Molecular testing for *A. lumbricoides* also failed to reveal sequence data with our *A. lumbricoides* primers. Importantly, *A. lumbricoides* is more susceptible to vermifuges (Kumm et al., 2010; Leles et al., 2010). The Piraino 1 clergyman certainly had access to medicines and even had medicinal botanicals in his digestive tract at the time of death (Piombino-Mascali et al., 2013).

Like whipworm, pinworm is considered to be a common crowd disease that is propagated through poor sanitation, and it is currently the most common intestinal helminth worldwide. *E. vermicularis* eggs are rarely seen in fecal analyses, because infected individuals typically shed eggs in only about 5% of stool samples (Garcia, 2007). This is because eggs are deposited on the perianal folds, cause itching, stick to fingers, and thereby contaminate the local environment. The unique pinworm lifestyle means that most infections will go undetected using traditional fecal analysis, which may be why eggs were not seen during previous microscopy examinations of the Piraino 1 coprolite sample. Without molecular techniques, the *E. vermicularis* co-infection in this individual would not have been identified.

Several types of commercially available immunological assays have also been useful in diagnosing parasites in archaeological samples (for example, see Faulkner, 1991; Gonçalves et al., 2004; Le Bailly et al., 2008, 2014; Morrow and Reinhard, 2016). None of the immunological tests utilized in this study of the Piraino 1 coprolite yielded positive results. If this individual had infections of *G. duodenalis, C. parvum,* or *E. histolytica*, then antigens did not survive intact enough to be detected by these tests, which show 98.5%-100% sensitivity with relatively fresh samples, according to the manufacturer. Given that primers specific to these parasites also failed to amplify, while primers for *T. trichiura* and *E. vermicularis* showed at least some genetic preservation in this sample, it seems reasonable to assume that this individual most likely did not suffer from these additional parasites.

In conclusion, this study reveals that the Piraino 1 clergyman had a co-infection of at least two parasitic intestinal helminths at the time of death. Both of these parasites are transmitted under poor hygienic conditions and are commonly found in archaeological samples across

the globe. This study also reaffirms the importance of collaborative studies that utilize multiple techniques to gather as much information as possible about the lifestyle and health patterns of individuals from archaeological contexts.

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CRediT authorship contribution statement Amanda Rollins: Conceptualization, Formal analysis, Investigation, Validation, Writing - original draft, Writing - review & editing, Funding acquisition. **Krystiana Krupa:** Investigation, Writing - review & editing. **Georgia Millward:** Investigation, Validation. **Dario Piombino-Mascali:** Resources, Writing - review & editing. **Karl Reinhard:** Conceptualization, Resources. **Frederika Kaestle:** Conceptualization, Investigation, Resources, Supervision.

Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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