Palaeoparasitology and palaeogenetics: review and perspectives for the study of ancient human parasites

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SUMMARY

While some species of parasites can be identified to species level from archaeological remains using microscopy (i.e. *Enterobius vermicularis*, *Clonorchis sinensis*), others can only be identified to family or genus level as different species produce eggs with similar morphology (i.e. *Tænia* sp. and *Echinococcus* sp.). Molecular and immunological approaches offer the possibility to provide more precise determination at the species level. They can also identify taxa when classic parasite markers such as eggs or cysts have been destroyed over time. However, biomolecules can be poorly preserved and modern reference DNA is available only for a limited number of species of parasites, leading to the conclusion that classic microscopic observation should be combined with molecular analyses. Here we present a review of the molecular approaches used over the past two decades to identify human pathogenic helminths (*Ascaris* sp., *Trichuris* sp., *E. vermicularis*, *Fasciola* sp. etc.) or protists (*Giardia* sp., *Trypanosoma* sp., *Leishmania* sp. etc.). We also discuss the prospects for studying the evolution of parasites with genetics and genomics.

Key words: Palaeoparasitology, palaeogenetics, helminth, protist, microscopy, PCR, next-generation sequencing.

INTRODUCTION

The study of ancient parasites, known as palaeoparasitology, is important for understanding the health status of past populations, their lifestyle and associated medical conditions. The investigation of the life cycles of parasites through the analysis of the markers preserved in archaeological samples can bring to light invaluable anthropological and ecological information (Dutour, 2013; Reinhard *et al.* 2013). For instance, it can highlight food practices when species associated with the consumption of undercooked or raw meat or fish are identified (for example, *Tænia* sp. or *Diphyllobothrium* sp.). Living conditions, waste and sanitation management can be evaluated when geohelminth remains such as *Ascaris* sp. or *Trichuris* sp. are retrieved in ancient human settlements. Proximity with animals can also be a source of parasite transmission to humans, such as for flukes *Fasciola* sp. or *Dicrocoelium* sp. (Le Bailly and Araujo, 2016). Since the first histological observation of *Schistosoma haematobium* by Sir Mark A. Ruffer in Egyptian mummies dated to the 20th dynasty (Ruffer, 1910), the microscopic analysis of eggs is the most usual approach to diagnose helminths preserved in historical contexts. Examination of the shape, size, the presence of opercula, caps and ornamental features of the egg shell, allows for a relatively accurate determination of the residues to the family or genus level. Identification at the species level is possible (for example, *Enterobius vermicularis*, *Clonorchis sinensis*, *Schistosoma mansoni* and *S. haematobium*) but uncommon as eggs from many species from the same genus can present similar morphological characteristics (for example, the eggs from the family Tæniidae: *Tænia* sp. and *Echinococcus* sp.). However, the specific determination would result in a more far-reaching understanding of the health status of the ancient populations under study and the host–parasite relationship.

To overcome this limitation, it can be appropriate to use immunological or molecular tools to identify the species precisely. Immunological diagnosis was performed for the first time in the late 1980s with the detection of *Giardia intestinalis* oocysts in a coprolite from the American site Big Bone Cave, dated to 2200 Before Present (BP) (Faulkner *et al.* 1989), using immunofluorescence. Since the 2000s, *G. intestinalis* and other intestinal protozoa of medical importance, such as the human pathogenic amoeba *Entamoeba histolytica* or the coccidia *Cryptosporidium parvum*, have been identified several times in remains from the Americas, Europe and the Middle East using Enzyme-Linked Immunosorbent Assay (ELISA) (Gonçalves *et al.*

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2002, 2004; Le Bailly *et al*. 2006, 2014; Mitchell *et al*. 2008; Le Bailly and Araujo, 2016). Using immunochromatography, such as the ParaSight™-*F* test, the detection of *Plasmodium* sp. (Miller *et al*. 1994; Cerutti *et al*. 1999) in Egyptian and Sudanese mummies (4150 years BP) has been reported with up to 35–40% positivity for malaria. In these cases, however, unspecific reactions of the antibodies used in the test have raised questions about the reality of the recovered frequencies of the disease (Bouchaud *et al*. 2000). The sole detection of a helminth using immunological techniques was reported by Deelder *et al*. (1990), with the detection using ELISA of *S. haematobium* from an Egyptian mummy dated to 5150 years BP. PCR combined with molecular hybridization experiments have been performed on Chilean mummified tissues dated to 2000 BP, leading to the identification of *Trypanosoma cruzi* (Ferreira *et al*. 2000).

In 2001, the first ancient DNA (aDNA) sequences from the helminth *Ascaris* sp. and the protist *T. cruzi* were published (Loreille *et al*. 2001; Madden *et al*. 2001). Since then, approximately 20 publications concerning palaeoparasitology have reported the detection of parasite aDNA. The vast majority of them targeted one to three taxa by PCR, followed by Sanger sequencing. Molecular hybridization, random amplified polymorphic DNA (RAPD) and polymerase chain reaction (PCR) have also been used to identify parasites such as *Trichuris* sp., *Ascaris* sp., *E. vermicularis* and *T. cruzi* (Guhl *et al*. 1999; Iñiguez *et al*. 2003; Jaeger and Iñiguez, 2014). Visualization of the PCR or RAPD products with electrophoresis gives interesting and quick information, but amplicon sequencing needs to be performed to assure the specificity of the results. Indeed, archaeological samples can be considered as environmental samples, colonized by numerous species of bacteria, fungi, viruses, etc. Public genetic databases used to verify the specificity of DNA primers and probes, do not list all the genomes of these organisms. These uncharacterized organisms may present similar electrophoresis profiles to the targeted species. If they are present in the sample, they can lead to false-positive conclusions. DNA from these untargeted organisms amplified by the enzymatic reaction can cause chimerical molecules due to jumping PCR, promoted by template damage, a characteristic of aDNA (Pääbo *et al*. 1990). This can also lead to false-positive results when sequencing is not performed.

Next-generation sequencing is not commonly used for the analysis of ancient human parasites since only three published works propose this approach. Our paper presents molecular palaeoparasitological works from the past 15 years. Table 1 summarizes the articles that we discuss in this review. We will also expose the benefits of high-throughput approaches and the perspectives for palaeogenomics and parasites.

PALAEOPARASITOLOGY AND SAMPLES

Whether they are colonized with bacteria, viruses, or fungi, the majority of the remains studied with palaeogenetics (bones, teeth or coprolites, for example) belong to a single-living organism. On the other hand, samples studied in palaeoparasitology are more complex, since the analyses are often performed on sediment sampled from cesspits, occupational layers, ditches or latrines, and are potentially made up of a mixture of organic matter. This mixture can originate from several individuals, humans and animals, all living together in villages or settlements. Palaeoparasitological remains associated with individuals, such as coprolites or mummified organs, are scarce, especially in Europe (Bouchet *et al*. 2003; Appelt *et al*. 2016). Accuracy issues in the diagnosis of ancient parasites with microscopic approaches can occur due to the difficulty in distinguishing human parasites from animal parasites in many cases. For example, with Taeniidae, it is not possible to distinguish eggs from the genus *Tænia* sp. (*T. saginata*, *T. solium* and *T. asiatica*, which may infest humans) from eggs from the *Echinococcus* sp. genus (eggs from *E. multilocularis* and *E. granulosus* can be found in canidae or felidae feces). This is also the case for many nematodes belonging to the Strongylidea order (Le Bailly *et al*. 2008). Moreover, if various strains of a species are present within a mixed sample, it is not possible to assess this situation solely with microscopic analysis.

MOLECULAR PALAEOPARASITOLOGY

Molecular palaeoparasitology, i.e. the genetic determination of ancient parasites, can overcome the problem of mixed samples and the diagnostic limitations of microscopy. It is possible to target genes that will allow for the discrimination of taxa with similar egg characteristics. Moreover, with the careful choice of genetic targets, it is possible to distinguish taxa at the species level. Accurate taxonomic determination would be more precise and highlight important parasitological, anthropological, ecological and archaeological information. Indeed, helminths present complex life cycles involving various intermediate hosts. A better understanding of interactions between the final and intermediate hosts could be possible with specific identification. For instance, discrimination between the *Tænia saginata* and *T. solium* tapeworms would indicate the human consumption of undercooked beef or pork, respectively.

The molecular approach can also identify parasites even when their eggs are destroyed or damaged due

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Table 1. Palaeoparasitological findings confirmed with sequencing approaches. The review has been established based on publications available in 2016

Sequencing approach	Parasites	Location	Age (BP)	References
PCR + Sanger sequencing	*Ascaris* sp.	Namur, Belgium	600	Loreille *et al*. (2001)
		Santa Cruz de Tenerife, Espagne	200	Botella *et al*. (2010)
		Chile and Brazil	8800	Leles *et al*. (2008)
		Korea	558–40	Oh *et al*. (2010)
		Viborg, Denmark	932	Søe *et al*. (2015)
		Belgium	650	Appelt *et al*. (2014)
	Trichuris sp.	Czech Republic	250	Myšková *et al*. (2014)
		Viborg, Denmark	932	Søe *et al*. (2015)
	Enterobius vermicularis	Chile and USA	6060–1050	Iñiguez *et al*. (2003, 2006)
	Fasciola sp.	Viborg, Denmark	932	Søe *et al*. (2015)
	Clonorchis sp.	Korea	265	Shin *et al*. (2013)
		China	2117	Liu *et al*. (2007)
	Echinostoma sp.	Peruaçu Valley, Brazil	560	Leles *et al*. (2014)
		Andea, Chile	1925–75	Madden *et al*. (2001)
	Trypanosoma cruzi	Peru and Chile	9000–100	Guhl *et al*. (2014)
		Brazil	1390	Fernandes *et al*. (2008)
		Germany	500–300	Panzer *et al*. (2014)
	Plasmodium sp	Abydos, Egypt	3450–2450	Nerlich *et al*. (2008)
		Abusir el Meleq, Egypt	3014–1650	Lalremruata *et al*. (2013)
	Leishmania sp.	Brazil	300	Novo *et al*. (2015)
		Abydos, Egypt and Nubia	4000–450	Zink *et al*. (2006)
		Chile	500–1000	Costa *et al*. (2009)
	Pediculus humanus	Chiribaya	925	Raoult *et al*. (2008)
	Encephalitozoon sp., *Enterocytozoon* sp., *Cryptosporidium* sp., *Giardia* sp.	Czech Republic	250	Myšková *et al*. (2014)
Next-generation sequencing	*Plasmodium* sp., *Toxoplasma* sp.	Egypt	2810–1826	Khairat *et al*. (2013)
	Ascaris sp., *Tænia* sp., *Dicrocoelium dendriticum*, *Fasciola hepatica*, *Enterobius vermicularis*, *Trichuris* sp., *Diphyllobothrium* sp.	Several archeological sites		Côté *et al*. (2016)

to taphonomic processes (Leles *et al*. 2008; Côté *et al*. 2016). Discrepancies between microscopic and palaeogenetic analyses due to the heterogeneous distribution of eggs and DNA within the sediment samples can be expected. If different subsamples from the archaeological remains are independently processed with both approaches, the likelihood of identifying taxa with the genetic approach only or with microscopy only is high. Moreover, the DNA and the eggs in the sediment are subject to various biological, chemical and physical factors that affect their preservation (Levy-Booth *et al*. 2007; Morrow *et al*. 2016). For these reasons, it has been suggested that palaeoparasitological samples should be analysed with a complementary approach combining microscopy and genetics (Cleeland *et al*. 2013; Appelt *et al*. 2014; Côté *et al*. 2016).

The determination of new genetic variants is also possible with the molecular approach. Phylogenetic analysis would then provide precious information on past diversity and cross-transmission events between humans and animals, such as during the domestication process of animals. Reference DNA sequences are required to proceed with PCR and sequencing leading to species identification. Thus, palaeogenetic analyses are confined to species for which genetic references are available. This strongly limits the number of taxa that can be targeted, in particular for animal parasites. Moreover, the morphological identification of modern specimens prior to genetic characterization must be accurate. An example of the impact of taxonomic misassignment has been exposed by Cleeland *et al*. (2013). The misidentification of *Physaloptera* sp. as *Contracaecum spiculigerum* due to the immature stage of development of the reference specimen was reported in that paper. Finally, phylogenetic analysis can also be limited by the poor representativeness of modern intraspecific variants. In 2013, most of the mitochondrial sequences from *Ascaris* sp. in Genebank were obtained from specimens from China or the USA. In that case, discrimination

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between ancient single-nucleotide polymorphisms (SNP) and modern biogeographic SNP can be laborious.

PALAEOGENETICS AND HUMAN GASTROINTESTINAL HELMINTHS

Between 2001 and 2015, six human gastrointestinal helminths were targeted by PCR after the observation of intact eggs under a light microscope. The soil-transmitted nematode *Ascaris* sp. (roundworm) was the most intensively analysed. This helminth has a similar life cycle to the whipworms from the genus *Trichuris*. Co-infestations with these two nematodes are frequent when food and water are contaminated with human feces. *Ascaris lumbricoides* and *Ascaris suum* are described as species that infest humans and pigs, respectively. However, genetic discrimination based on mitochondrial genes is not possible. Differences in the mitogenomes of the two species appear to be associated with geographical distribution rather than signifying specific variants (Liu *et al*. 2012). Recent works suggest that the cytochrome c oxidase- (cox 1) haplogroup can cluster *Ascaris* with human or pig hosts. However, the modern distribution of this nematode is complex. Indeed, specific mitochondrial haplogroups cluster with a specific host in endemic regions but zoonotic infection appears to be frequent in Europe (Betson *et al*. 2014). Due to the degradation of aDNA molecules (Pääbo, 1989), the 450 base pair (bp) fragments used to determine the cox 1 haplogroup may not be appropriate for palaeogenetic studies. aDNA from the roundworm *Ascaris* has been identified in French, Spanish, Korean and Danish remains sampled from coprolites, from the surface of pelvis or from egg concentrations processed in sediments (Loreille *et al*. 2001; Leles *et al*. 2008; Botella *et al*. 2010; Oh *et al*. 2010; Søe *et al*. 2015). The cytochrome b and/or the 18S rDNA genes were targeted in five studies and do not allow for the identification of the species. The mitochondrial haplogroup 07, typical of parasites from pigs in Europe, has been described from the Danish sediments from which eggs from the human whipworm *Trichuris trichiura* were also determined. This is indirect evidence of an anthropic infestation, since the sample was a mixture of various structures, including a waste-dumping pit that may have been used for the disposal of organic matter from humans and animals (Søe *et al*. 2015). *Ascaris* and *Trichuris* co-infestations have also been described for the microscopic analysis of remains from Korea, Denmark and the Czech Republic and amplification of the 18S rDNA gene confirmed contamination by *T. trichiura* (Oh *et al*. 2010; Myšková *et al*. 2014; Søe *et al*. 2015).

The human pinworm, *E. vermicularis*, was also studied using palaeogenetics. Infestations by this parasite are frequent in modern populations. The light and fragile eggs are easily transmitted by handling contaminated objects. Preserved eggs were described in archaeological remains from the Americas (Fry and Moore, 1969; Araujo *et al*. 1985; Gonçalves *et al*. 2003) and the Middle East (Nezamabadi *et al*. 2013; Paknazhad *et al*. 2016). Molecular determination based on the 5S rDNA intergenic region was performed on 27 coprolites from Chile and the USA, dated between 4110 before Christ (BC) to 900 anno Domini (AD) (Iñiguez *et al*. 2003, 2006). Strikingly, with the exception of Herrmann (1988), who analysed latrines from medieval Germany, no microscopic study has yet characterized pinworm eggs in European samples. Thus, no palaeogenetic diagnosis based on the prior observation of eggs has been performed for this region, raising questions concerning the early distribution of pinworms.

Fasciola sp. and *Clonorchis* sp. are flukes that cause distomatosis in animals and humans. *Fasciola hepatica* and *F. gigantica* are distributed worldwide, while most of the infections by *C. sinensis* are in China (Wu *et al*. 2012). Palaeogenetic analyses have been performed on Danish remains, identifying *F. hepatica*, in addition to *T. trichiura* and *Ascaris* sp. (Søe *et al*. 2015). The environmental origin of the remains does not allow for clear conclusions concerning animal or human infestation by the fluke. Internal Transcribed Spacer (ITS) 1–2, and cox 1 sequences of *C. sinensis* have been obtained on a Chinese corpse buried in 167 BC (Liu *et al*. 2007) and in a medieval Korean mummy (Shin *et al*. 2013). While sequences of ITS1 in the latter showed that 100% identify with modern references, 15 SNP have been identified in the Chinese remains. Combined with previous modern genetic studies (Lee and Huh, 2004), ITS1 appears to be a good marker to study the phylogeny of this Asian fluke. *Echinostoma* sp., a fluke with worldwide distribution, has also been described in a Brazilian human coprolite (560 BP) by the sequencing of a fragment of cox 1 gene and ITS1 region. Resolution at the species level identified the species *E. paraensi* (Leles *et al*. 2014).

PALAEOGENETICS, PROTISTS AND OTHER PARASITES

The vector-borne unicellular parasite *Trypanosoma* sp. is widely distributed throughout the world. The causal agent of Chagas disease, *T. cruzi*, is confined to Central and South America and affects ~10 million people (CDC-parasites). With a reservoir made up of over 100 species of animals and with 12 species of insects acting as vectors, colonization by this parasite appears to predate the arrival of humans in the region.

Morphological indications of infestation reported on mummies, such as the mega visceral syndrome

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(Rothhammer *et al*. 1985), and molecular analysis, suggest that humans have been infected by *T. cruzi* for a long time. The sequencing of kinetoplastic DNA from four Chilean mummies dated between 25 and 1875 AD provides the first ancient sequences of *T. cruzi* (Madden *et al*. 2001). Subsequently, 283 samples from Peru and Chile were analysed by hybridization of PCR products without sequence analyses. The infection rate reached over 40% of the samples, for a period of 9000 years (7050 BC–1850 AD) (Aufderheide *et al*. 2004). Phylogenetic analyses were performed on a set of 17 mummies from the same location using mitochondrial and nuclear gene datasets. Six different genotypes were described (TcI, TcII, TcIV, TcV, TcVI and TcBat), corresponding to oral-transmission strains (Guhl *et al*. 2014). TcI was also identified in a Brazilian mummy (560 AD) (Fernandes *et al*. 2008). These results highlight the high level of genetic diversity of *T. cruzi* and raise questions about vectorial and/or oral routes of infestation in the past. A multidisciplinary study, including palaeoradiology, palaeogenetics, forensics, and isotope analysis, was performed on an unidentified mummy conserved in a German museum, suggesting a South American origin for the corpse. Palaeopathological investigations by radiology showed evidence of Chagas disease, confirmed by the palaeogenetic identification of the parasite (Panzer *et al*. 2014).

Plasmodium sp. is a blood parasite that infects vertebrates and four species can cause malaria in humans. Due to its vector-borne transmission cycle involving the insect *Anopheles*, this disease is restricted to tropical and subtropical regions below an altitude of 1500 m (CDC-parasites). While classic molecular markers for *Plasmodium* in ancient remains have led to unspecific sequences, the *Plasmodium falciparum* chloroquine resistant transporter gene was identified in two Egyptian samples (Nerlich *et al*. 2008). These remains, dating from the New Kingdom to the Late Period in West-Thebes, were associated with osteopathological signs of anaemia, which supported the genetic results. Palaeopathological analyses were also performed on mummies from the Egyptian necropolis of Abusir el Meleq (1064 BC–300 AD), with molecular evidence of co-infection with *Mycobacterium tuberculosis* and *P. falciparum* (Lalremruata *et al*. 2013). A 196-bp fragment of the apical membrane antigen gene was targeted, followed by genotyping of the merozoite surface protein-1 gene. Many cases of co-infection by tuberculosis and malaria were reported at the end of the 19th century/beginning of the 20th century.

More than 20 species of *Leishmania* can infest humans, causing visceral, cutaneous or mucosal forms of leishmaniasis. *Leishmania tropica*, *L. major* and *L. aethiopica*, *L. infantum* and *L. donovani* are species that affect populations from the Old World. *Leishmania mexicana* and *L. (V.) braziliensis* are complex species commonly affecting populations from the New World (CDC-parasites). In 2009, DNA from skulls with unspecific signs of infection have been analysed using PCR and sequencing. The identification of L. donovani in this area of San Pedro de Atacama (Chile) was unexpected. This arid desert at high altitude is unlikely to have been endemic and the presence of *Leishmania* can be associated with the migration of people from an endemic neighbourhood (Costa *et al*. 2009). Palaeogenetic analysis reported the infection of a Brazilian mummy dating to the Colonial Period by *L. tarentolae* (Novo *et al*. 2015). This species is considered non-pathogenic for humans, but infected geckos from the Old World. This result raises questions as to an ancient occurrence of the disease, but also as regards the ecology of the parasite, since this *L. tarentolae* was not detected in modern Brazilian reptiles. *Leishmania donovani* was identified in remains from Abydos (Middle Kingdom, Egypt, 2050–1650 BC) and Upper Nubia (550–1500 AD) (Zink *et al*. 2006). Due to the sandfly vector and woodland distribution, *Leishmania* is presumed to be non-endemic in ancient Egypt. Since no molecular evidence of *Leishmania* infection has been retrieved from the Pre-to Early Dynasty (3500–2800 BC) and in the Late Period (2050–500 BC), ancient Egyptians are thought to have been contaminated during trade with Nubians.

Analyses of sediment from archaeological structures preserved in Prague (18th–19th centuries) indicate infestation by several intestinal parasites, including *Trichuris* sp., *Capillaria* sp., *Ascaris* sp., *Diphyllobothrium latum*, *Dicrocoelium dendriticum*, *F. hepatica*, and from the Opisthorchiidae, Taeniidae and Heterophyidae families (Myšková *et al*. 2014). Molecular analyses detected the whipworm *Trichuris* sp., the microsporidia *Encephalitozoon* sp. and *Enterocytozoon bieneusi*, the Apicomplexa *Cryptosporidium* sp., and the flagellate *Giardia* sp. Cysts from these organisms are rarely preserved over time, thus they do not provide a source or potential protection for ancient biomolecules.

Finally, the human louse *Pediculus humanus* was collected from hair belonging to a Chiribaya mummy from Peru (1025 AD cal.). Sequence analysis and phylogeny based on cox 1 gene identified the phylotype A of *P. humanus*, the most widespread phylotype in modern populations (Raoult *et al*. 2008).

NEXT-GENERATION SEQUENCING APPROACHES

Palaeogenetic analyses based on classic Sanger sequencing of PCR products can give an interesting overall picture of the presence of pathogenic agents in individuals from ancient populations. It is also

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an appropriate approach to support the hypothesis of parasitic infestation when macroscopic signs are observed. However, methodological issues can arise for the analysis of multiple samples and when several taxa are targeted. This would drastically increase the number of PCR amplification experiments, which can be expensive and time-consuming. Moreover, depending on the taxonomic identification level that can be achieved with the primer pairs, infestation with multiple species from the same genus can be difficult to diagnose with cloning techniques and Sanger sequencing. While the sediment collected can be a mixture of organic matter from several individuals and/or animals, then this approach can draw an incomplete palaeopathological picture. Next-generation sequencing technologies can generate a large number of sequences and thus provide better parasitological diagnosis.

As it is not limited by the selection of species targeted by PCR, shotgun sequencing may offer a more complete diagnosis of the pathogens (parasites and bacteria) preserved in the sample. The study of intestinal microbiota by metagenomic analysis can lead to the identification of gastrointestinal parasites. Metagenomic sequencing was performed on a sample collected from an Egyptian mummy (806 BC–124 AD) and led to the identification of the pathogens *Plasmodium* sp. and *Toxoplasma gondii* (Khairat *et al*. 2013). *Toxoplasma* sp. is an intracellular parasite with a complex life cycle, involving the Felidae, and mice or birds as intermediate hosts. Humans can be infected by eating undercooked meat from infected animals or by consuming food or water contaminated with cat feces. Cats were a cult animal in ancient Egypt and the identification of *T. gondii* in a human mummy indicated proximity with this animal. Metagenomics can thus provide important knowledge on intestinal microbiota and pathogens that affected ancient populations. However, this approach may be not appropriate for genetic studies focusing on parasites. A relatively high throughput is necessary to detect ancient parasitic molecules mixed with high numbers of prokaryotic, viral and fungal DNA molecules. For example, while microscopy and PCR identified *Ascaris* sp. in a 14th-century coprolite, metagenomic analyses performed on the same sample yielded ~ 107 000 reads, with 32% corresponding to known sequences, and none have been assigned to *Ascaris* (Appelt *et al*. 2014). Again, the association of several approaches, combining microscopic identification with molecular determination, will result in more reliable palaeoparasitological identification.

Depending on the research project, the sequencing of amplicons with medium throughput technology could provide more information than Sanger sequencing of individual PCR products. Even though this approach restricts the diversity of the taxa that can be identified, its low cost can be an alternative to metagenomics. Based on this type of approach, 14 archaeological sites were analysed with a method called aMPlex Torrent. Out of the 17 targeted species of gastrointestinal helminths, nine taxa were identified (*Ascaris* sp., *T. saginata*, *T. solium*, *T. asiatica*, *D. dendriticum*, *F. hepatica*, *E. vermicularis*, *T. trichiura*, *Diphyllobothrium* sp.) (Côté *et al*. 2016). This flexible method appears to be a suitable approach for screening taxa from multiple samples.

PROCEDURES FOR aDNA ANALYSES

Since the first report on the analysis of aDNA fragments from a 140-year-old Quagga, an extinct subspecies of horse (Higuchi *et al*. 1984), palaeogenetics raises interest from the scientific community. Due to the characteristics of aDNA [minute amount of short fragments diluted in environmental DNA, and biochemical modifications of nucleotides due to taphonomic processes (Pääbo, 1989)], authenticity of the DNA sequences also became a focus of interest. In 2000, a list of criteria of authenticity has been published (Cooper and Poinar, 2000) and as we consider that this list is still relevant and topical, we briefly present the most important points.

A separated and isolated laboratory dedicated for the processing of ancient samples (preparation of the remains, extraction and purification of DNA and preparation of the reagents for enzymatic reactions) is essential to avoid contamination with modern DNA. Multiple negative controls, such as parallel mock purifications and non-template PCR controls should always be included during the preparation of DNA and PCR. Positive control using modern DNA should be avoided. Positive results should be repeatable using the same DNA extract, and when possible, re-extraction from the same samples should confirm the results. Finally, quantitative characterization of the DNA extract should be performed to assess the starting number of templates and to test the molecular behaviour: due to the nature of the ancient molecules, PCR products longer than 1000 bp are not usual. PCR amplification efficiency should be inversely correlated to the product size to reflect this characteristic. When possible, independent laboratories should proceed to the replication of the complete experiment (from the extraction to the sequencing analysis). In our opinion, two additional criteria need to be mentioned. Prevention of contamination, especially when working with human remains, should start during archaeological excavations. Contaminant DNA can come from the archaeologist or the anthropologist who excavate and handle the remains. Palaeogeneticists and archaeologists should collaborate to establish good field practice. Finally, as commercial reagents are

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potential sources of modern DNA, we suggest proceeding to the decontamination of buffers, enzymes and primers, especially when targeting ancient human or bacterial DNA (Champlot *et al*. 2010).

CONCLUSIONS/FUTURE DIRECTIONS

Over the last century, gastrointestinal helminths from archaeological sites have been described based on the preservation of fossilized remains. While their eggs can be preserved for a long time, with evidence of parasitic infestation in palaeontological coprolites (Poinar and Boucot, 2006; Dentzien-Dias *et al*. 2013; Hugot *et al*. 2014; Silva *et al*. 2014), taphonomic processes can affect their morphological features, leading to imprecise taxonomic identification. Five taphonomic factors have been described as having an impact on egg preservation: abiotic, contextual, anthropogenic, organismal and ecological factors (Morrow *et al*. 2016). Badly preserved or destroyed eggs can lead to an underestimation of the occurrence of taxa. This was the case for the *E. vermicularis* pinworm in Europe. Eggs have been described in a single archaeological site in Germany (Herrmann, 1988), while positive molecular results have been obtained from various European locations since the Neolithic period (Côté *et al*. 2016). Genetics can thus provide an accurate diagnosis of species, even when the eggs are poorly preserved. The biomolecules are also affected by taphonomic processes or can be adsorbed on mineral particles (Blum *et al*. 1997). This phenomenon could lead to false-negative results, in particular if the molecular methods used for detection are not sensitive enough. Besides, for Apicomplexa parasites, such as *Toxoplasma* sp. or *Plasmodium* sp., cysts are rarely preserved. Genetics appears today to be the only approach for the identification of these pathogens.

In Côté *et al*. (2016), we studied 25 samples from 14 archaeological using microscopic determination and genetic analyses. For each samples, using only one approach would have resulted in a less accurate parasitic diagnose. Complementary studies combining palaeogenetics with microscopic identification are thus the best method to provide the most complete and accurate diagnosis possible.

Genetic determination is still limited by the presence of modern reference sequences. The lack of modern molecular analyses performed on animal parasites or rare human parasites, such as the capillariid group, limits the diversity of species that can be characterized in archaeological contexts. Intraspecific genetic variants, including geographical variants, are impossible to distinguish from ancestral states if few modern sequences are available. This can also cause limited phylogenetic conclusions. Until recently, *Ascaris* phylogeny was only based on Asian and American samples and did not allow for discrimination between animal and human species, if such differences truly exist. Extensive analyses of mitochondrial and nuclear markers of worms from multiple locations allow for a better understanding of the natural history of this nematode (Betson *et al*. 2014). Including ancient sequences in the analyses would increase our knowledge of its evolution and may indicate past cross-transmission events between humans and swine.

As discussed, new sequencing technologies have been underemployed for molecular palaeoparasitology up until now. Recently, target-enrichment capture methods based on DNA hybridization have been used for the reconstruction of ancient prokaryotic genomes (Bos *et al*. 2011; Maixner *et al*. 2016). Palaeogenomics appears to be the next step for a better understanding of the evolution of parasites. For instance, ancient genomes could highlight genetic adaptations due to the migration of populations, to host shifting, to climatic changes, and to the use of antiparasitic drugs.

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