2008

Paleoparasitology: Perspectives with New Techniques

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Published in *Revista do Instituto de Medicina Tropical de São Paulo* 40:6 (Nov/Dec 1998);
doi: 10.1590/S0036-46651998000600006

INVITED REVIEW

Paleoparasitology:

Perspectives with New Techniques

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Received: 19 May 1998
Accepted: 22 June 1998

SUMMARY
Paleoparasitology is the study of parasites found in archaeological material. The development of this field of research began with histological identification of helminth eggs in mummy tissues, analysis of coprolites, and recently through molecular biology. An approach to the history of paleoparasitology is reviewed in this paper, with special reference to the studies of ancient DNA identified in archaeological material.

KEYWORDS: Paleoparasitology, Coprolites, Parasites in archaeological material, Paleopathology, Archaeoparasitology, Mummies

RESUMO

Paleoparasitologia: perspectivas com novas técnicas
Paleoparasitologia é o estudo de parasitos encontrados em material arqueológico. O desenvolvimento deste campo da pesquisa teve início com a identificação de ovos de helmintos em tecidos mumificados, análise de coprólitos e, recentemente, através da biologia molecular. Neste artigo faz-se uma breve revisão da história da paleoparasitologia com referência especial aos estudos de ADN antigo (ancient DNA) em material arqueológico

INTRODUCTION

In 1987 the first description of techniques used for the recovery of parasite eggs from archaeological materials was published[54]. Since that time, the exploration of archaeological remains has expanded and new techniques have been devised. The most important development during the past decade has been the application of molecular biology techniques for the recovery of ancient parasite DNA. Also, chemical digestion of archaeological sediments was introduced for the effective recovery of parasite eggs from all types of archaeological deposits. We review here the history of techniques within the context of the theoretical perspectives and the study goals of paleoparasitology.

The Pioneering Period - Paleoparasitology (also archaeoparasitology) is the study of parasites in ancient material. The first report of ancient parasites is Ruffer's (1910) diagnosis of *Schistosoma haematobium* eggs in kidneys from Egyptian mummies. RUFFER[57] used histological sectioning and staining for the identification of the eggs. Although a few other pioneering papers appeared in the first half of this century recording parasite eggs in archaeological material[28,49,64,65], the field really developed in the 1960s and was finally named in 1979[15,16].

Initially, parasitologists analyzing coprolites tried different flotation techniques[62,65]. These are effective in unconsolidated sediments where parasite eggs are well preserved. However, standard clinical techniques were not effective when parasitologists turned their attentions to coprolites. Coprolites are desiccated and sometimes mineralized feces. To analyze coprolites, the trisodium phosphate rehydration technique was introduced[9]. This technique was adapted from methods used to rehydrate desiccated zoological specimens in museums[67]. Further experiments demonstrated that the trisodium phosphate technique was effective when applied to coprolites[61]. These studies showed that trisodium phosphate at 0.5% concentration in aqueous solution results in the reconstitution of the eggs and larvae of parasitic worms. It was shown that the egg shells and anatomical features of the larvae such as the esophagus and intestine are visible after application of trisodium phosphate. Thus, the application of this simple technique allows for the microscopic diagnosis of parasitic worms.

In the late 1960's and early 1970's, there was a burst of paleoparasitology studies as the trisodium phosphate technique was widely applied to coprolites from Utah, Arizona, Colorado, and Nevada. These pioneering efforts can be characterized as a "discovery" phase. During this time, it was demonstrated that parasitism dates back to remote times and that prehistoric humans were hosts to a wide variety of parasites. In 1967, Aidan Cockburn pointed out that coprolite studies had a great potential for defining the evolution of infectious disease in relation to cultural evolution[11]. He urged parasitologists to interpret their data from an epidemiological perspective. Cockburn's message reached paleoparasitologists in North and South America. Still, it took several years before his recommendations were followed and broader paleoparasitological interpretations were scarce through the 1970's and 1980's[1,22,34,43]. But, by the late 1980's, paleoparasitological data began to be interpreted to a greater extent.

Paleoepidemiology, 1980-1997

After the pioneering and discovery periods in paleoparasitology, researchers began struggling with new methodological questions. The consistent problems for paleoparasitologists are the diagnosis of the zoological origin of coprolites found in archaeological layers, and the diagnosis of the parasites themselves. A reference collection of desiccated feces of living mammals belonging to a national park in northeast Brazilian was prepared for comparison with the coprolites found at archaeological sites in the same region[10]. The method shows good results when it can be applied. However, it is a long and tedious process to survey all animals to cover all the possibilities of fecal morphology.

Similar procedures are encountered regarding parasites. When the host is known, parasite check-lists are very useful. Morphometric parameters must be examined, as proposed by experimental paleoparasitology[12,35]. However, extinction and changes in the local fauna must be considered[16]. Also, when eggs of a parasite not previously known to exist in humans are found, careful evaluation of the infection must be done to determine if a true case of parasitism is represented. This problem was encountered with acanthocephalan eggs in coprolites from Utah and Arizona[22,40]. The eggs were identified as *Moniliformis clarki*. Analysis of the dietary constituents of the coprolites and the biology of *M. clarki* showed that this was a parasite of humans. Therefore, careful paleoparasitological analysis showed that *M. clarki* was a common parasite of prehistoric Indians before indigenous dietary patterns changed. In other cases, careful analysis reveals "false parasitim" when eggs of a parasite are consumed and passed through the intestinal tract without hatching[71].

During these two decades paleoparasitology advances relied on morphological parameters of parasite remains. Ligth microscopy has been the main tool for scientists. However, other techniques including immunology and electron microscopy have been introduced. HORNE experimented with transmission electron microscopy (TEM)[33]. Although HORNE did not recommend that TEM replace light microscopy, TEM did allow for the identification of internal parasite egg structures. Scanning electron microscopy (SEM) is a useful diagnostic tool. In certain cases, fungal spores, and especially pollen can be confused with parasite eggs when only light microscopy is applied. SEM allows for the examination of surface features that can be used to distinguish them[54]. Also, SEM is a very useful tool for the characterization of helmith larvae[2]. Immunological tests have significant potential for paleoparasitology. FOUANT was the first to

apply immunological analysis to parasite remains[21]. Her application of ELISA to possible *Entamoeba histolytica* cysts proved negative. Immunofluorescence stains were successfully applied to identify *Giardia lamblia* cysts in coprolites from Kentucky[14]. In our opinion, immunology has a great potential for identifying parasite remains.

Once the methodological problems discussed above were resolved, great advances in paleoparasitology occurred. Parasite infections were identified in various locations and a picture began to emerge of the distribution of parasites and prehistoric migration routes of their hosts[3,4]. It can be said that paleoparasitology has reached a stage of metamorphosis from a descriptive stage to a period of true contributions to the pathoecology of parasitism.

Quantitative studies also showed interesting epidemiological patterns. Ancient hunter-gatherers were shown to have a reduced parasite fauna relative to agricultural populations[50]. Also, hunter-gatherer parasitism is dominated by zoonotic species whereas agricultural populations had more human-specific parasites. Subsequent comparison of parasite prevalence in coprolites with bone lesion prevalence (porotic hyperostosis) in skeletons showed a correlation between parasitism and anemia[51]. Comparisons of the pathoecology of prehistoric agricultural villages showed that the level of parasitism was dependent on the local ecology, sanitation patterns, and house style[52]. Detailed studies of house type through 10,000 years of prehistory in the southwest United States have shown that pinworm prevalence is related to the style of house construction which affects air flow[54].

A different technique was developed in the 1980s that led to the recovery of data from soils from archaeological sites. In 1986, REINHARD et al.[54] adapted palynological techniques used for the recovery of pollen to the recovery of parasite eggs. By using treatments with hydrochloric acid, hydrofluoric acid, and zinc bromide heavy density solution, and adding *Lycopodium* tracer spores to the sediments, parasite eggs could be concentrated and quantified from any archaeological sediments. In subsequent years the technique was used on latrine soils from many archaeological sites and also on sediments from gardens. In these analyses, the widespread distribution of parasites was documented for urban sites in Israel and North America.

Molecular biology and ancient parasite DNA

Today, new perspectives were opened with the introduction of molecular biology techniques and some their applications are reviewed in this paper.

During the last ten years infectious diseases started to be diagnosed using technologies based on nucleic acid. Parasite diagnosis is the last field of clinical microbiology to use these techniques, and the role that they can play in epidemiology, prevention, and treatment of parasitic diseases is enormous. The probe based on nucleic acid for the detection of parasites consists of the use of a reporter molecule of DNA to detect specific sequences of parasite DNA or RNA. The parasite in the sample is lysed and the nucleic acid is released and denatured.

The polymerase chain reaction (PCR) is based on the replication *in vitro* of the double-helix molecule of DNA. It is used to amplify a segment of DNA situated between two regions of a known sequence. Two oligonucleotids are used as primers for a series of reactions catalyzed by

an enzyme, DNA polymerase. PCR is the synthesis of millions of copies of a specific DNA segment.

Ancient DNA (aDNA) or ancient RNA (aRNA) are nucleic acids recovered from archaeological, paleontological or museum specimens. In a broader sense it can be applied to any nucleic acid recovered after death when the autolysis process was started[29].

Hybridization was the first technique used to recover DNA from archaeological material[68]. The first molecular clone of animal DNA was prepared using the skin of an extinct zebra[30]. PÄABO and WILSON et al. worked with human DNA of archaeological origin[43,69]. However, relatively large amounts of DNA are needed for the hybridization technique.

In 1985 the polymerase chain reaction (PCR) was incorporated. It is sensitive and can be performed easily, permitting the use of small nucleic acid fragments from human, other animals, and plants.

The PCR technique was described by Kary MULLIS in 1985[41], and SAIKI et al. improved it[58,59]. The technique was then adapted for archaeological material[44,45]. Ancient DNA was then amplified from human bones and mummified tissues [24,25,26,27,32,46]. The importance of PCR for archaeology was reviewed, showing the perspectives and limits of the new technique[8,47]. Sex determination and the study of phylogenetic relationships in ancient populations were shown to be possible with this approach[38,63].

Protozoal infection in ancient material

Helminths are the most common parasite finds in archaeological material. Eggs and larvae can be well preserved by desiccation, or, at times, even by mineralization. Even before the use of rehydration technique, parasite eggs were found in archaeological material.

Protozoan infections are not easy to identify in archaeological remains. *Entamoeba coli* cysts were recorded in the intestinal contents of a Peruvian mummy[49], and protozoan cysts were detected in human coprolites dated 1800 BP (before present)[70]. *Eimeria* cysts were found in deer coprolites dated 9000 BP, from Brazil[17].

Tissue protozan infections were even more difficult to diagnose. Small ceramic statues found in pre Columbian burial sites suggested cutaneous leishmaniasis lesions[66], and histopathological examinations of mummified bodies showed lesions identified as Chagas' disease[56]. *Trypanosoma cruzi* was found using electron microscopy and the diagnosis was confirmed by histochemical techniques, in a Peruvian mummy[20].

Recently, seven Chilean mummies were found to be positive for *T. cruzi* by the PCR technique[22]. This findind confirmed the presence of Chagas' disease since at least 4,000 years ago in the Andean region.

Ancient molecular biology: experimental research

1.Mucocutaneous Leishmaniasis

Many pre Columbian Andean populations used ritual burials where small clay statues (huacos) accompanied the body. Some of them show destructive nose and lip lesions, similar to those of mucocutaneous leishmaniasis[31,48].

Leishmaniasis prevalence is related to some ecological aspects of hosts, vectors and forest reservoirs[13]. Natural infection is common among Marsupialia, Edentata, Rodentia, few carnivores, primitive Ungulata, and primates including man[37].

Rodents are very important for the epidemiology of mucocutaneous leishmaniasis[19] but the infection is usually asymptomatic[36]. However, the importance of the role of forest rodents in the maintenance and transmission cycle of the disease is not sufficiently understood.

In an attempt to study the role of rodents in the transmission cycle of leishmaniasis, PCR was applied to a sample of taxidermized rodents collected 50 years ago.

To test the technique, experimentally infected mice were taxidermized with the same technique used in the collection[39]. The assay was conducted on 11 young mice (Balb/c) infected with *Leishmania amazonensis* (106 promastigotes/ml) in the foot. After two months, 25 mg were collected from each animal (three controls were used) and DNA was extracted. PCR followed the method of Sambrook (1989) and the QUIAGEN protocol (1996).

The results were positive for *Leishmania* in all infected samples and negative in noninfected mice, and allowed the study in the museum collection.

Sixty thousand specimens of rodents were taxidermized and stored in the National Museum (Universidade Federal do Rio de Janeiro). The collection was the result of the plague campaign from 1941 to 1975, covering the entire country. Some rodents were captured in endemic leishmaniasis areas. When possible, the material was collected where lesions were present and from two known endemic regions. From Baturité - Ceará state, the following species were examined: *Oryzomys elliurus* (1), *O. subflavus* (10), *Kerodon rupestris* (1), *Trichomys apereoides* (2), *Galea spix* (2), and *Zygodontomys pixuna* (4). And from Ilha Grande - Rio de Janeiro state, *Rattus norvegicus* (4), *O. lamia* (6), *Proechymus dimidiatus* (7) and *Phyllomys sp* (1). (number of specimens examined)

Taking care to avoid contamination, DNA isolation and purification were performed using the commercial QIAamp-tissue kit (QUIAGEN, 1996).

Standard procedures were followed using taqDNA polymerase and positive and negative controls were used for each PCR reaction. Hybrydization was performed to confirm the results[60].

Five of 39 animals were found to be positive for *Leishmania.* The results showed that the PCR technique can be applied to epidemiological studies of the past, and also to the diagnosis of leishmaniasis in mummies with suggestive lesions.

Chagas' disease

Fifteen years ago it was observed that archaeologists were bitten by triatomines (*Triatoma brasiliensis*) when they were doing copies of rock art in the archaeological site of Pedra Furada, Piaui state, northeastern Brazil. At that time it was supposed that the ancient artists could have been infected by *Trypanosoma cruzi* 20,000 years ago. Unfortunately, no technique was available to diagnose the microorganism in the skeletons found at the site.

PCR makes this study possible today, and we began this research line in our Laboratory of Paleoparasitology.

The first step was to test experimentally desiccated infected material. *T. cruzi* infected mice were desiccated at 39^0C and PCR was used to identify *T. cruzi* DNA. The results obtained suggest that the application of this technique to *T. cruzi* detection in archaeological material was possible[6,7].

After testing the technique, we applied it to mummified tissues collected from 7 mummies from Atacama (Museo Arqueologico de San Pedro de Atacama, Chile). The first results showed positive PCR, but hybridization is in process. The second step is to test the technique in bones and tissues from the archaeological sites of Piauí state, Brazilian northeast.

Other parasitic infection

Research for ancient DNA in archaeological material has also been investigated for bacteria and viruses in coprolites and mummified tissues. DNA of *Shigella flexneri* was found in pre-Columbian coprolites from Chile showing potentialities for this field in paleoparasitology. Bacterial and viral DNA in archaeological material is an open field for phylogenetics and the evolution of diseases and the future is promising.

The Future of Paleoparasitology

In the past, the innovation and application of new techniques led to significant contributions of paleoparasitology to the general understanding of the pathoecology of parasitism. For example, the wide application of the trisodium phosphate technique to coprolites in the past led to profound developments in the understanding of the evolution and distribution of parasitic disease. Also, the application of soil digestion to archaeological sediments led to the definition of the nature of urban parasitism. Similarly, future discoveries in pathoecology of parasitism depend on the broad application of new techniques to new materials. What are the potential techniques and materials?

Since 1994, there has been a new emphasis on mummy studies. Parasitologists have been quick to begin devising new techniques for application to mummies. The advantage of mummies is that it is possible to recover adult and larval stages of parasites, and also that parasites of somatic tissue can be recovered[53]. Also, ectoparasites can be analyzed from mummies[5,51]. Thus, it is probable that mummies will be the main focus of paleoparasitology technique development. This is already evident in the discussion presented above. Researchers in Brazil are working intensively on the development of analysis techniques for mummies.

The techniques that will be useful in mummy studies will include DNA and immunological analyses. Tests for antibodies and antigens, and searches for distinctive parasite proteins are underway or are planned. The application of immunological test has proven useful in coprolites and will undoubtedly be useful in mummies. Also, aDNA studies will be expanded. The preservation of tuberculosis aDNA sequences in Peruvian mummies, and the experimental application of this technology to leishmaniasis and trypanosomiasis indicate that application of PCR technology will result in the recovery of parasite aDNA.

We also feel that newer developments in microscopy will facilitate the study of parasites in mummies. The laser confocal microscope will prove to be useful in characterizing lesions caused by ancient parasites. The development of the environmental SEM may also prove important since this technology does not require extensive preparation of specimens by critical point drying. Therefore, more delicate samples from mummies may be used for parasite study.

Thus, the future of paleoparasitology will expand from coprolites and soils to include mummy studies. In this way, a greater diversity of species, including protozoa, helminths and arthropods will be studied and the ecology of their diseases elucidated.

Acknowledgments

This work was supported by CNPq and PAPES/FIOCRUZ. We thank Paulo Cesar Martins for technical support in the laboratory.

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