J. Biol. Res. 2005 n. 1 - Vol. LXXX - Rubbettino - Soveria Mannelli

Detection of Plasmodium falciparum ancient DNA in Egyptian mummies

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KEY WORDS: Malaria, *Plasmodium falciparum,* ancient DNA, mummy.

Introduction

The detection of ancient microbial DNA offers a new approach for the study of infectious diseases, their occurrence and host – pathogen interaction in a diachronic view, over the centuries. Moreover, the data that are obtained from the skeletal and mummified tissue may represent an important completion of contemporary phylogenetic analyses of pathogens. The study of microbial DNA in ancient remains contributes to the understanding of transmission and spread of infectious diseases, and potentially to the evolution and phylogenetic pathways of pathogens (Zink, 2002).
Malaria is one of the most widely distributed and severe diseases affecting *Homo sapiens*. Malaria has been recognized as an important parasitic disease of humans for millennia, having been described by the early Egyptians in the third millennium B.C.
Malaria is of great interest in epidemiological and paleopathological studies, since it has been, and still is an important disease for human populations.
Despite of the introduction of control and prevention programs in many parts of the world, the impact of malaria on human populations continues to increase. Recent estimates suggest that 1.5 billion persons live in areas of the world where malaria is an endemic disease. The number of infected humans exceeds 500,000,000 and 1-2 million persons die each year.
In certain areas of the world, this infestation is one of the main causes of death in the pre-reproductive population. Thus the malaria has been a very important factor that influenced the fitness of ancient peoples. All agree that the indirect proof of the antiquity of the origin of malarial protozoans is the presence of numerous genetic adaptations, which required a very long time, that led to speciation of *Plasmodia* (*P. falciparum, P. vivax, P. ovale, P. malariae*) and parallel to polymorphisms in human erythrocytes.
The size of the *P. falciparum* genome is about 23 MB, composed of 14 chromosomes, that range in size between 0.7 - 3.3 MB. There is estimated to be around 5300 genes in the *P. falciparum* genome.
The first attempt to investigate the presence of *P. falciparum* in ancient mummies was the application of immunological methods (Miller et al., 1994; Cerutti et al., 1999; Rabino Massa et al., 2000).
The first paper that describes a PCR method (Polymerase Chain Reaction) for identifying *P. falciparum* DNA in a skeletal specimen is by Taylor et al. (1997). A hemi-nested PCR was used, that employs a set of primers designed to anneal to a portion of the 18s ribosomal RNA genes. Taylor et al. amplified successfully malarial DNA from a rib bone from a man who died in 1937 with anemia believed to be caused by malaria. The sequencing of the PCR products revealed 98% sequence identity with the 18s ribosomal RNA gene of modern *P. falciparum*. Taylor attempted to apply the same PCR method to analyse a rib from another subject who died of malaria in 1936, as well as a medieval skeletal specimen from Spain and the Granville mummy from ancient Egypt. He failed to produce any amplifications. Sallares et al. (2001) discussed previous studies of malaria in old specimens (both for the proteome and the genome of *P. falciparum*). They attempted the amplification of *P. falciparum* DNA from five skeletons coming from Lugnano infant cemetery in Rome, believed to be the result of an epidemic of malaria. Only one yielded PCR products which were shown by sequencing to be 98% identical to the *P. falciparum* 18s ribosomal RNA gene sequence.

Materials and methods

The aim of this report is to show the preliminary results of the detection of *P. falciparum* ancient DNA in mummified and skeletal specimens. The specimens were taken from seven Egyptian mummies belonging to Marro's Collection in the Museum of Anthropology and Etnography in Turin. These mummies are pre-dynastic (3,200 B.C.) and they come from the Egyptian site of Gebelein (Fig. 1). This was a primitive fertile agricultural area situated near the Nile river, an ideal malarial habitat. The standards of selection for these mummies were the macroscopic and radiographic observation of morphological skeletal changes (porotic hyperostosis, *cribra orbitalia*) produced by iron deficiency anaemias, which might be caused, directly or indirectly, by malaria (Angel, 1966). These modifications could also have a

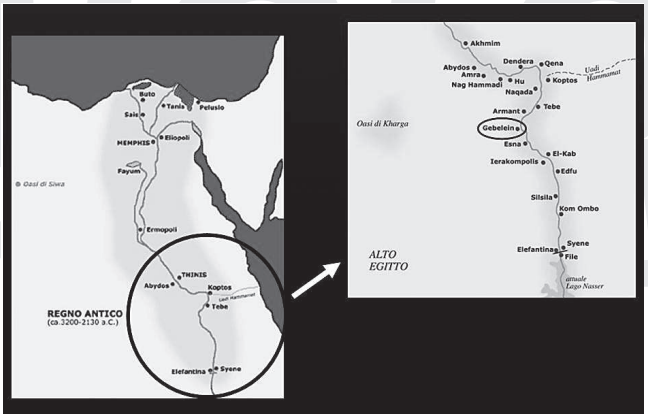

Fig. 1 - Geographical map of the Gebelein area in Egypt.

variety of other causes completely unrelated to malaria. This is a major area of controversy among physical anthropologists. Nevertheless the advent of biomolecular archaeology offers a new and more direct approach to investigating such problems.

In a previous study, mummified tissue specimens, taken from the same mummies, were analysed with an immunological test for detecting *P. falciparum* and the results were positive (Cerutti et al., 1999). The state of these mummies is very good. The malarial proteome (HRP-2 antigen) has been well preserved so that we addressed the analysis of the genome of *P. falciparum* in the same mummies.

The collecting of the specimens consisted in the removal of mummified muscular tissue, dental pulp and an internal portion of femur. The DNA extractions (Fig. 2) were performed by three different methods (using the Chelex, the phenol-chloroform and the Invisorb Forensic kit) in order to compare the amount and the purity of DNA and to establish the most efficient method to extract ancient DNA.

Since the ancient DNA is very fragmented, from literature on modern malarial DNA studies, we selected different sets of primers that amplified small size products (no more than 300 base pairs). Several Polymerase Chain Reactions were performed as reported in the Figure 2. The amplifications anneal to portions respectively of the 18s ribosomal RNA and EBA-175 genes of *P. falciparum*.

The 18s ribosomal RNA genes are very suitable for ancient DNA analysis because of their high copy number in *P. falciparum*. Besides these genes are well known markers in evolutionary studies about relationships between the different species of *Plasmodium*.

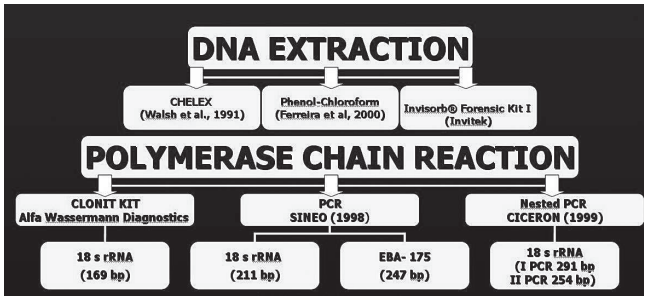

Fig. 2 - DNA extraction and PCR methods performed to investigate P. falciparum ancient DNA (the references are reported in brackets). The genes and the relative size of PCR products are reported in the end line.

In Figure 3, it is reported that the nested PCR method was used as suggested by Ciceron et al. (1999). This method consists of a double amplification. The first set of primers amplify a fragment of 291 base pairs. The second step is the amplification of the first PCR products with a second set of primers that anneal to the internal portion of the target DNA, in order to obtain a major number of copies. The nested PCR method may be very suitable for ancient DNA studies because it optimises the amplification from a small amount of DNA.

In all the amplifications, there were included blank controls (without addition of DNA to the PCR reagents) and negative controls (DNA of healthy individuals). The positive control was never used in the laboratory where the PCRs of ancient DNA were performed.

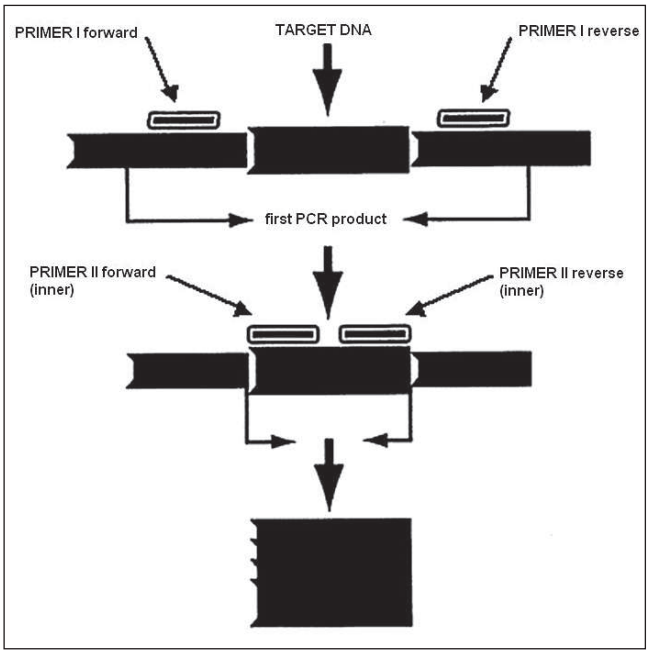

Fig. 3 - Nested PCR method.

The amplification products were submitted to electrophoresis in a 2% agarose gel with Etidium Bromure and visualized on a transilluminator. The PCR products that were not visible were dried, resuspended in lowTE and submitted to electrophoresis again.

The preservation of human genomic ancient DNA has been evaluated in the same specimens by the amplification of regions of the beta globin and ACE genes.

All precautions were taken in order to avoid contamination. Separate rooms were used for performing DNA extractions, setting up PCRs and post PCR operations. Plugged pipette tips were used. All reagents were autoclaved. Furthermore, the experiments were repeated twice in two different laboratories.

Results

Some of the electrophoretic runs on the agar gel are reported in the Figure 4. These preliminary results indicate that the DNA extracted with the Invisorb® Forensic Kit I

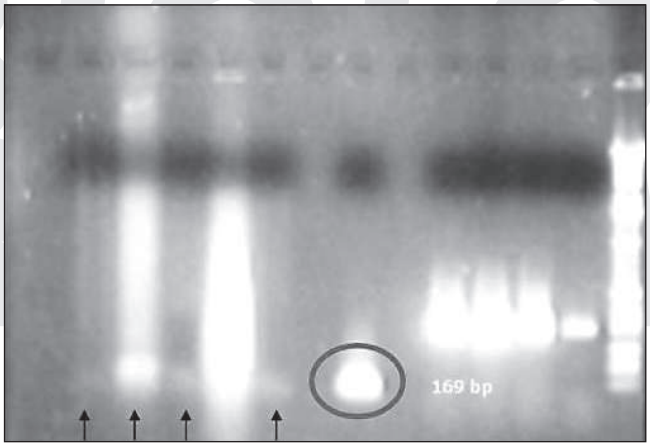

Fig. 4a - Electrophoresis TBE 2% agar gel: (A) DNA extracted with Invisorb® Forensic Kit I and amplified with Clonit Kit.

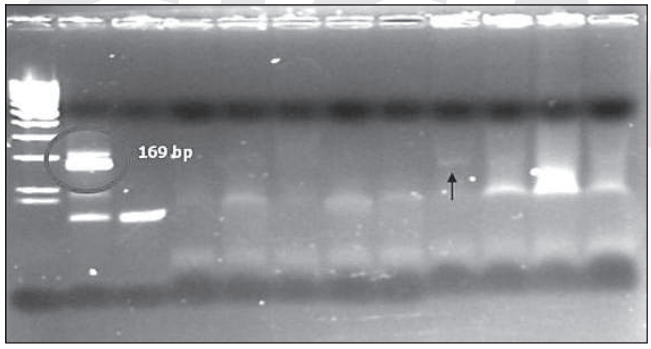

Fig. 4b - PCR products lyophilized, resuspended in low-TE and electrophoresed again.

was successfully amplifyed with the CLONIT KIT for four mummies. The three amplification products, that were not visible, were dried and resuspended in lowTE and submitted to electrophoresis again. In this way another sample showed a band of 169 bp. The positive control, that is indicated in the red circular line (Fig. 4a, b), was extracted from the fresh blood of a patient with malaria diagnosed by an immunological test (Amedeo di Savoia Hospital). The amplification was not performed in the laboratory where the ancient DNA was analysed.
The phenol chloroform and the chelex extractions were not successful and they gave inhibition in amplification.
The specific identity of the PCR products will be verified by sequencing the fragments and they will be compared with the sequence of the 18s rRNA gene of *P. falciparum* and other organisms.

Conclusions and Perspectives

On the basis of Taylor and Sallare's previous experiences, it cannot be assumed that a band appearing on the agar gel actually is the *P. falciparum* DNA, until the PCR products have not been sequenced. So these results should be considered absolutely preliminary. Considering the previous immunologically positive results, our prospects seem hopeful for the sequencing of the *P. falciparum* DNA. Ribosomal RNA genes are highly conserved. Our perspectives in the molecular palaeopathology of *P. falciparum* is to find alternative targets in other parts or the genome. In particular, we are looking for more specific targets, like the genes for cell-surface proteins, which might be expected to be more variable and distinctive given their function in interacting with the human immune system.
In spite of these preliminary positive results, the study of *P. falciparum* ancient DNA on a large scale shows some limitations. In fact, some malarial infections are asymptomatic with very low levels of parasitaemia. Even when death does occur, it is frequently caused not by malaria alone, but by the synergistic interactions with other diseases. Such synergistic interactions may be very frequent in ancient populations. Besides it is possible to have very low concentrations of parasitaemia (malarial DNA) even in modern cases, especially if the patient has inherited resistance (for example beta globin mutations) or alternatively if the patient has acquired immunity following repeated infections in childhood.
Nevertheless, malaria infection has a systemic nature, associated with a vast number of parasites. In each parasite, multiple copies of rRNA genes are present making potentially possible the detection of *P. falciparum* ancient DNA in well preserved human bones and mummies.

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