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Preliminary Findings on the Paleomicrobiological Study of 400 Naturally Mummified Fuman Remains from Upper Nubia

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Abstract

We present 400 mummies excavated from two early Christian burial sites at Kulubnarti, between the 2nd and 3rd cataracts of the Nile in Northern Sudan, prior to the flooding caused by the Aswan Dam. One site was on an island in the Nile dated from 550-750 AD. The other was on the Nile western bank and was in use from c.750-1500 AD. Due to the exceptionally dry climate many of the remains were naturally mummified. Analysis of diet, via chemical examination of hair and of coprolites, had indicated possible deficiencies in vitamins B6, B12, folacin and vitamin C, suggesting iron deficiency. The presence of criba orbitalia, a pathological lesion of the roof of the eye socket (orbit), also suggests iron deficiency anaemia but may also be caused by inflammation. Anaemia is found in several infectious diseases, and severe iron deficiency increases susceptibility to disease. Tuberculosis was widespread in ancient and Roman Egypt, and there was historical contact with Upper Nubia via the Nile. The presence of Acacia pollen in coprolites suggested the possibility of leishmaniasis, as these trees are the habitat of the sand fly vector of the protozoan pathogen. The area is known today for the many infectious diseases afflicting its inhabitants, but were these present in antiquity? We have, therefore, undertaken a study of diseases in Kulubnarti. Initially we looked for evidence of tuberculosis and leishmaniasis. We anticipate shortly broadening the search to include brucellosis, malaria, hepatitis and West Nile fever viruses. Schistosomasis was considered but at this level the Nile flows swiftly and the intermediate host snail is not present so was at present not ocnsidered. Ribs were examined for Mycobacterium tuberculosis DNA, using nested PCR targeting a123 bp sequence on the repetitive element IS6110. Material from the heads of the long bones, possible bone marrow, was examined for Leishmania species using a PCR which amplifies a 119 bp-conserved region of the minicircle kinetoplast DNA. Initial results indicate that M. tuberculosis and Leishmania sp were present in both populations.

Introduction

Nubia has been described as «the corridor to Africa» and as the «connecting link» between civilizations of the Mediterranean and sub-Saharan Africa. These characterizations reflect the dynamic interaction of geography and behaviour that has shaped the politics, economy, and biology of Nubia's ancient populations. Several hundred human remains were removed from two burial sites at the medieval site of Kulubnarti; This was located approximately 128 km south of Wadi Halfa and just north of the Dal Cataract in the *Batn el Hajar*, meaning «belly of rock». This area has been described as an inhospitable environment, lunar in appearance and "the most barren and forbidding of all Nubian environments». One village was situated on the island of Kulubnarti and the other on the adjacent left bank of the Nile, representing ancient agricultural communities of the early

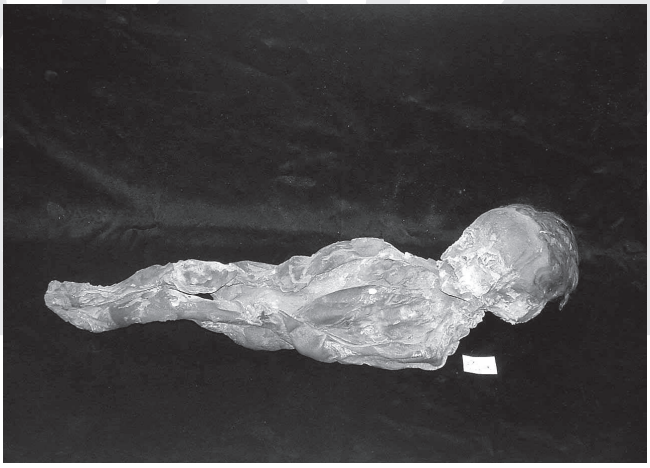

Fig. 1 - Well preserved child mummy

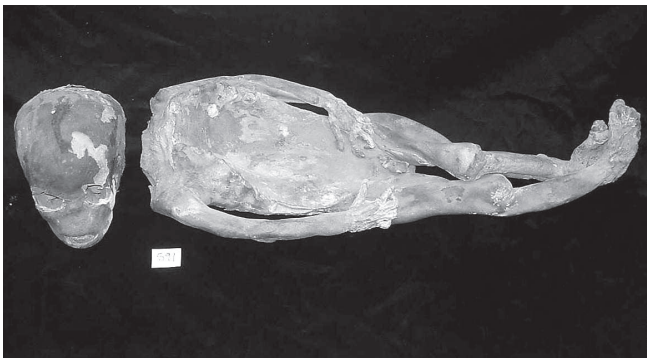

Fig. 1a- Another child mummy

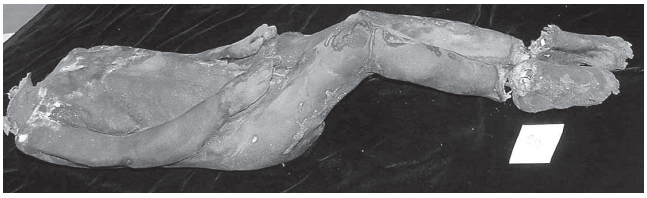

Fig. 2 - Adult mummy

(AD 550-750) and the late (AD 750-1450) Christian periods, interred in one of the driest environments on Earth (Van Gervan *et al.* 1995).

The Sudanese Nubian assemblage, consisting of over 400 naturally mummified individuals, is considered one of the best-preserved collections in the world (Figs. 1-1a-2). Hair, nails, skin, internal organs, and intestinal contents are present for many members of this community. Hair with intact cornrows, adults wrapped in burial shrouds, newborns with the umbilicus tied in twine, and even last trimester foetuses were preserved. The level of preservation can be gauged in figure 3, which shows what are believed to be red blood cell casts in bladder tissue. These biconcave discs are of the correct size but we do not exclude the possibility of spores, and work continues on these cells.

The excellent preservation of these mummified remains has provided the opportunity for macroscopic, microscopic, chemical, and genetic analyses to be conducted since their disinterment in 1979. Previous studies of fracture rates and degenerative disease have demonstrated a clear pattern of mechanical stress. Studies

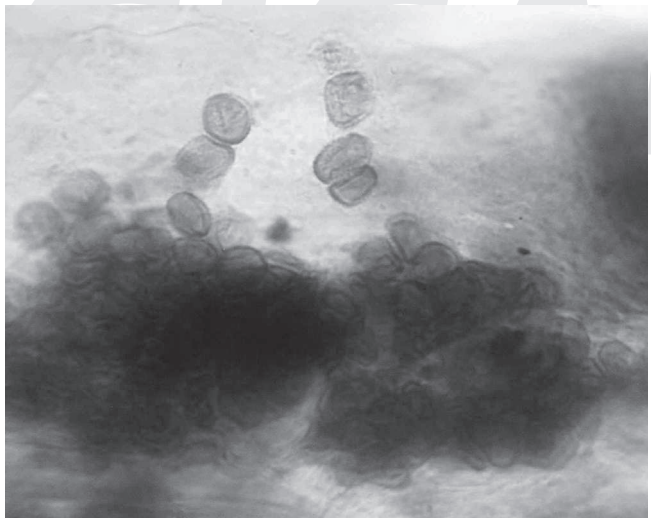

Fig. 3 - Suspected RBC in bladder wall

of bone growth, development, and ageing have also been an integral part of the ongoing research directed toward reconstructing the bio-cultural adaptations of this ancient community. Examination of skeletal remains has demonstrated a clear pattern of nutritional stress, iron and magnesium deficiencies, osteopenia and osteoporosis, deficiencies in vitamins C, B_6, B_{12} and folic acid, trauma (fractures of long bones, crania, hands and feet plus dislocations) and childhood stress syndrome, which consists of iron deficiency anaemia and growth interruption (Van Gerven *et al.* 1995). This was supported by the presence of *criba orbitalia*, a pathological lesion of the roof of the eye socket (orbit) that has been associated with iron deficiency anaemia but may also be caused by inflammation. Anaemia is found in conjunction with several infectious diseases, such as tuberculosis, leishmaniasis and helminthiasis.

The area is known today for the many infectious diseases afflicting its inhabitants but, were these present in antiquity? We have undertaken a study of diseases in this Kulubnarti, population initially looking at tuberculosis (TB), leishmaniasis, brucellosis, and malaria. We anticipate shortly broadening the search to include viruses such as hepatitis and West Nile fever.

Leishmaniasis is a parasitic disease caused by the haemoflagellate of the genus *Leishmania*. The dog, reservoir for *L. infantum* and *L. donovani*, has been domesticated for thousands of years and, along with the rodents' reservoir, probably spread from western Africa with migrations of man out of Africa (Nozais 2003). Today more than 21 species cause human infection, which is transmitted to humans through the bites of female sand flies belonging to 30 species. The disease manifests mainly in three types: the visceral, the cutaneous and the mucocutaneous leishmaniasis. The diagnosis of the visceral form is conventionally made by demonstrating the presence of the intracellular amastigote form of the parasite in aspirated fluid from the bone marrow, the spleen and, rarely, from the lymph nodes or the liver. Parasite demonstration and isolation rates from cutaneous and mucocutaneous lesions are rather poor due to low parasite load and high rate of culture contamination.

Molecular techniques targeting various genes of the parasite have also been reported, PCR being the most common molecular technique successfully used for diagnosis and for differentiation of species (Salotra *et al*. 2001; Singh and Sivakumar 2003, Maurya *et al*. 2005; Oliviera *et al*. 2005**)** Nowadays, leishmaniasis is endemic in some parts of Sudan. The sand fly vector of the visceral type of the disease, *Phlebotomus orientalis*, is positively associated with the presence of *Acacia seyal* and, it is of interest that Cummings (1989) recorded pollen of *Acacia* sp. in these coprolites. It has been proposed that the *Leishmania donovani* complex initially emerged from the *Acacia* forests in East Africa (Sudan and Ethiopia) before, or concurrent with, the emergence and spread of modern humans, and that the parasite may have subsequently followed the trail of its human hosts in their eventual diasporas (Ibrahim 2002). So the presence of leishmaniasis in early Christian Nubia is not unexpected. Tuberculosis was widespread in ancient and Roman Egypt (Zink *et al.* 2001, Donoghue *et al.* 2004) and historical contact with Upper Nubia via the Nile is proven.
Urinary schistosomiasis, caused by *Schistosoma hematobium*, is a serious disease of the inhabitants of the Nile valley. According to Kloos and David (2002), available information on the evolution of the parasite, the snail intermediate hosts and humans indicates that Schistosomasis originated in East Africa and became widely distributed in North Africa during prehistoric wet phases. Even though the Nile flows swiftly at this level, they consider that physical and human environment of this region provided increasingly favourable conditions for the transmission and spread of urinary Schistosomasis after the development of irrigation agriculture during the early Pharaonic period. Whilst our specimens have thus far yielded no morphological evidence of ant worm eggs especially not Schistosoma we do believe they must have been present especially in the irrigation canals despite the fact that the Nile at this level flowed to fast to allow the host snail to live in its watewrs.
Although the snail fossil record is poor in the ancient Nile valley and delta, *Bulinus truncatus* host snails were probably widespread, also indicated by their recovery from irrigated areas and wells elsewhere in the Middle East. According to Bouchet *et al.* (2003), emergence and dispersal of Schistosomasis along the Nile was confirmed by Harter (2003) in High Nubia by the finding of *S. mansoni* and *S. haematobium* eggs in shroud fragments (700-300 BC) and vegetable fibre plugs (1500 AD). Other evidence is its immunodiagnosis in some mummies from 35-550 AD in the Wadi Halfa riverine area near the Egypt-Sudan border (Miller *et al.* 1992), but these results are were provisional and await confirmation. More recently. *Schistosoma mansoni* DNA has been reported from ancient Egypt (H. Donoghue and C. Matheson, personal communication) and the organism detected by immunocytochemistry (Rutherford 1999).

Methods

We sampled the entire collection of 418 mummies using what we believe should become a standard piece of

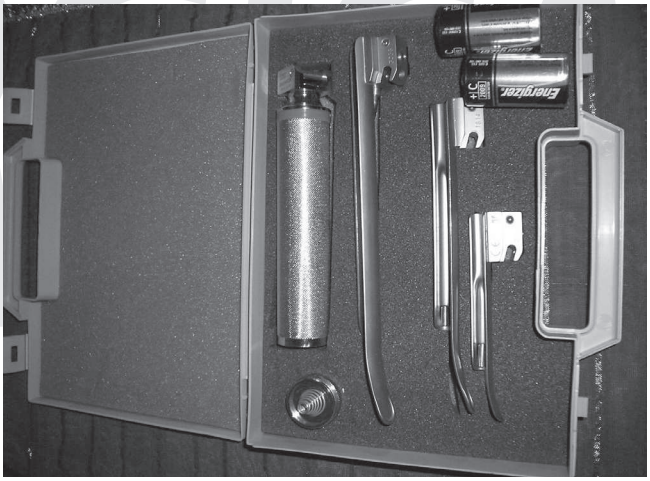

Fig. 4 - Portable laryngoscope

equipment for mummy studies, particularly in the field: a battery operated portable laryngoscope with a long arm (Figs. 4-5). This provides enough vision in the majority of mummies, allowing entry through a small portal or a natural or *post mortem* opening in the body (only tightly wrapped bodies, such as some Egyptian ones, may at times be unsuitable for this instrument). The biopsy forceps are disposable endoscopy forceps that most hospitals discard after one use. We had no trouble in obtaining them and then having them sterilised. They appear to last a long time and were still functioning after sampling over 400 mummies though we did have a dozen at our disposal so they could be properly cleaned between usages.
Initially some of the coprolite analysis was re-examined. The collected coprolites, were each associated with an individual body, thus providing age and sex information. In her Ph.D. research, Cummings (1989) had examined 48 samples, which provided a rich record of pollen, phytoliths, plant and fauna tissues for dietary analysis. All the coprolites and abdominal contents recovered from individual burials were presumed to be human by the very nature of their association. Questions involving the general health of the population may be answered through the examination of coprolites, and may be related to dietary intake and / or questions of disease or parasitic conditions. With the aid of molecular biology techniques, mainly PCR, new studies on the diet are being carried out in order to compare the results of DNA and microscopic analyses. No evidence of helminth ova have yet been obtained. A plausible explanation may be the presence of garlic, cloves and other natural vermicides / vermifuges in the diet, inferred from the coprolite examination. However, ova absence in the faeces is not proof of absence of helminthiasis in this population. Cummings noted in p. 83: «No evidence of parasite ova or eggs was obtained from coprolite analyses" Nematodes were expected to be recovered, however numbers in modern samples are low and detection is not always highly successful - this is not suggestive of the absence, but rather a methodological constraint.
In our molecular studies described below we observed full precautions as dictated by modern opinions on this subject

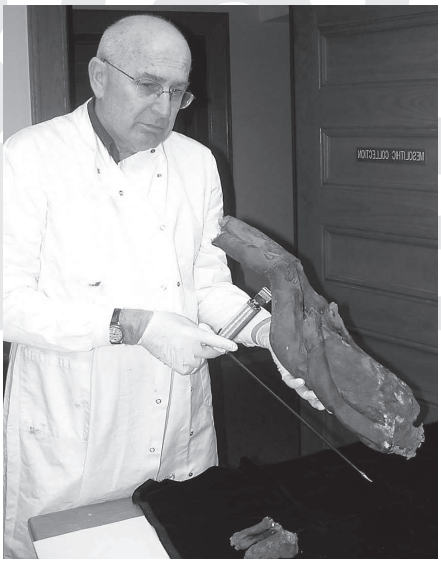

Fig. 5 - Actual sampling with laryngoscope

(See box 1). We also did repeat extractions and PCR amplifications on all our positive samples. The laboratories in London and Jerusalem performed independent verification tests on each other's samples.
The epiphyses of the long bones, primarily the heads of the humerus and femur, possibly containing bone marrow, were examined for *Leishmania* species. These bones had previously had a wedge remove for other tests, which facilitated access to the sampling site. Potential marrow sample was removed by scraping the internal surfaces of the bones, especially in the bonehead. The kinetoplast DNA in *Leishmania* spp. forms catenated circles of which thousands of copies are contained in each protozoan cell. This kDNA was targeted using a pair of oligonucleotides, (Rodgers *et al.* 1990) that produce a small amplicon of 116-120 bp published as 13a (5'-dGTGGGGGAGGGGCGTTCT-3') and 13b (5 -dATTTTACACCAACCCCCAGTT-3'). The internal transcribed spacer (ITS) region was also amplified and hybridized to characterize the *Leishmania* sp.
Visceral surfaces of mainly upper ribs from the mummies and partially skeletalised remains were examined for *Mycobacterium tuberculosis* DNA, using PCR targeting initially a 123 bp insertion sequence on the repetitive element IS*6110* (Eisenach *et al.* 1990) and, subsequently, a 92 bp nested sequence (Donoghue *et al.* 1998).

Results and discussion

Thus far, material from 103 individuals has been tested ; 70 of them had marrow samples and all were examined for TB and for leishmaniasis, respectively. The DNA was extracted using a modified guanidinium thiocyanate method (Boom *et al.* 1990 Spigelman *et al.* 2002) and purified with acidified silica beads (Höss and Pääbo 1993). PCR amplification was performed using Platinum Taq™ DNA polymerase (GIBCO) or Qiagen HotStar® *Taq* polymerase and reagents. This reflects the differing techniques of each laboratory involved. Nine of the 70 marrow samples were positive for *Leishmania*. As it has been suggested previously that the *L. donovani* complex initially emerged from the Acacia forests of East Africa at or before the emergence of modern humans in Africa (Pratlong *et al.* 2001; Ibrahim 2002) the presence of this disease in early Christian Nubia is not unexpected..
Tuberculosis was detected in approximately 30% of all samples from both populations. *M. tuberculosis* DNA was found in 43% of rib samples, with a distribution suggesting a higher incidence in children and adult females. We generally try and test the visceral surfaces of upper ribs as this is where primary lesion are most likely to occur and where there may be extension of disease onto the rib surface in particular.
Since the congress the UCL laboratory has found considerably more rib samples had TB (32%) than bone marrow samples (5%). We have now examined seventy-five samples from sixty-two individuals for tuberculosis. Twenty-nine of these were from the early Christian burial site, and thirty-three were from the later Christian burial site. The results show that there was a higher prevalence of TB in the late Christians (40%) than the earlier populations at Kulubnarti (34%). Reasons for this are unclear, but as this is a disease whose spread is facilitated by poor housing, overcrowding and lower standards of living, it is possible that in later years this population was under duress for some reason, leading to worsened living conditions, and thereby enabling the spread of disease. Younger inhabitants of Kulubnarti seemed to have suffered more from TB than their adult counterparts. In the early settlement 29% of children aged 0-5 were TB+ but 71% in the later settlement (though total number are rather low); aged 6-18 the figures were 50 %and 44%; for adults the figures were 33% and 41%. Considering gender, 36% of adult males and 46% of adult females were TB+ but this was not statistically significant.
As further data are obtained it is likely that some of the observations noted above will reach statistical significance. The high level of tuberculosis in children is clearly apparent, together with suggestive associations with the worsening prevailing socio-economic conditions in the later settlement.
In continuing the *Leishmania* work we have two aims:
We wish to estimate the leishmaniasis prevalence in the Kulubnarti and the western bank of the Nile populations in 550-750 AD and 750-1500 AD, respectively, to see if VL could have been a significant cause of morbidity and mortality in the area.
We wish to determine which *Leishmania* species was endemic in the two areas and if it is the same species that is still epidemic in the Sudan.
This is a preliminary report on ongoing work and we hope by the next congress to report on the entire collection.

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Box 1. Standard criteria for ancient DNA authentication (O'Rourke *et al* 2000):

- Complete physical isolation of work area
- Multiple control reactions
- Appropriate molecular behaviour
- Reproducibility within laboratory
- Cloning of PCR products
- Replication in independent laboratory
- Biochemical preservation of specimen
- Quantification of DNA template
- Examine DNA in animal bones at sites

Box 2. Precautions against cross-contamination:

- Protective clothing, gloves and filter-tips
- Thorough and frequent cleaning
- Modern DNA in class I safety cabinet in category

Box 3 laboratories under negative pressure

- Use of separate workstations and rooms
- DNA extraction, PCR set-up and post-PCR
- Solutions and pre-aliquoted PCR mix purchased