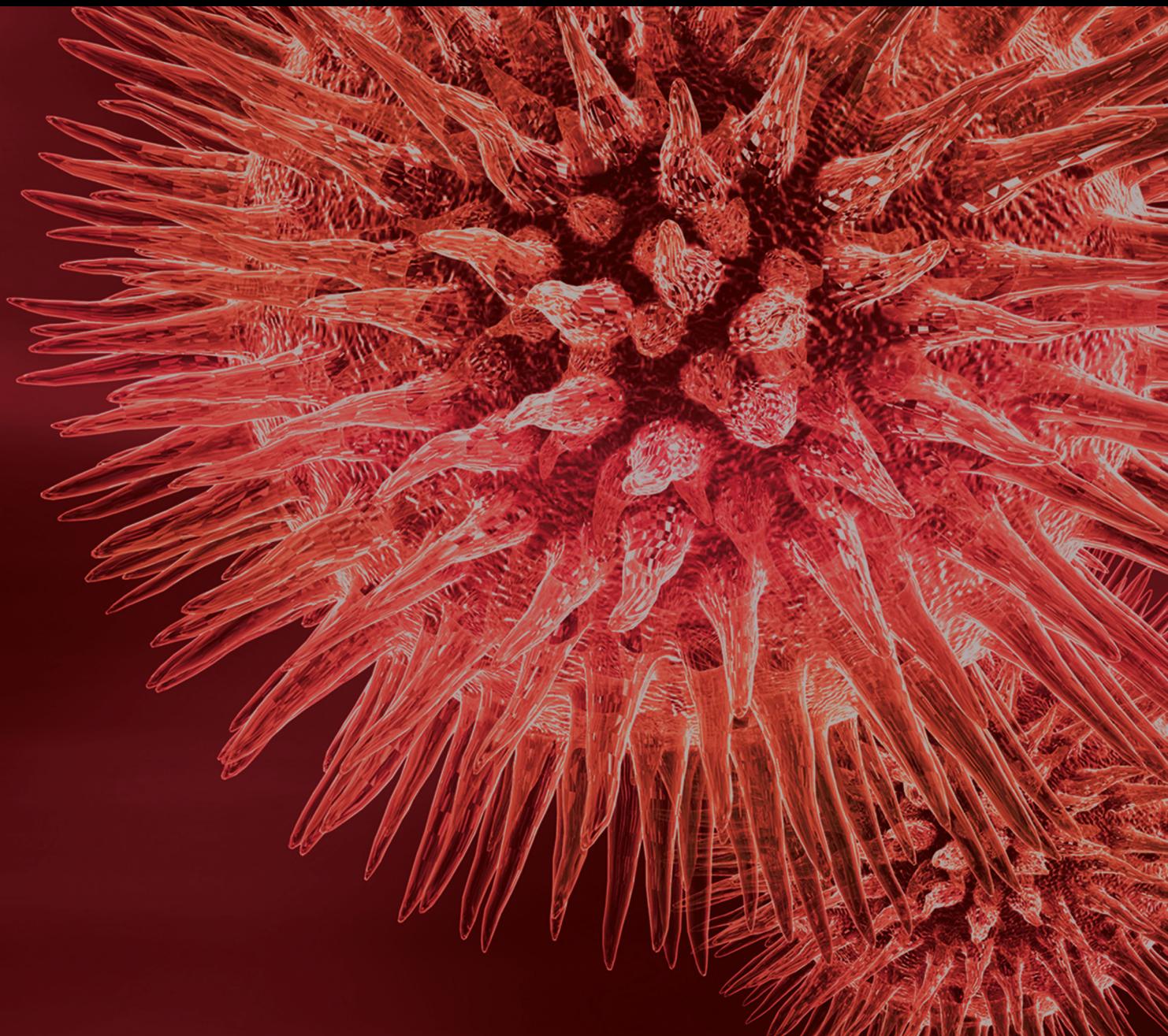


BioMed Research International

Current Trends in Studies of Ancient Diseases

Lead Guest Editor: Dong H. Shin

Guest Editors: Raffaella Bianucci, Robert D. Loynes, Hisashi Fujita,
and Myeung Kim





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Editorial

Current Trends in Studies of Ancient Diseases

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For decades, paleopathology, the science that investigates diseases and related conditions by examination of skeletal and soft-tissue remains, has allowed scholars to reconstruct historical populations both demographically and epidemiologically. Thanks to the recent advances in paleogenetics, knowledge on ancient human migrations, diets, and pathogens has been greatly augmented. More recently, the systematic pathographic approach to the study of ancient diseases has added further to the accumulated data on how ailments were diagnosed and treated in different social-cultural contexts. By combining the pathological evidence on skeletal and mummified remains with the analysis of ancient texts and literary sources, a holistic approach to the study of past morbidity conditions and related treatments can be applied. This special issue aims to provide examples of these new trends.

G. D. Al-Khafif et al. provided the first evidence of nonfalciparum malaria in the skeletal remains of Old Kingdom Egyptians from the Giza skeletal collection. Resorting to an immunochromatographic assay (RDT), they detected aldolase, the pan-malarial antigen, in 56% of analyzed individuals, and found no significant differences between wealthy officials and workers. As described in ancient texts, the annual Nile flooding tended to affect increased numbers of mosquito breeding sites; starting with the Dynastic Period, this trend was accelerated by the construction of irrigation systems. Archaeological and textual evidence indicates that

Ancient Egyptians protected themselves from the *Anopheles* mosquitoes; for instance, the bed of 4th Dynasty Queen Hetepheres I, the wife of King Snefru who ruled Egypt between c 2613 and 2589 BC, most likely had been designed to bear a protective bednet. Similarly, the use of herbal remedies for malaria (possibly garlic-based remedies) was reported by the Greek historian Herodotus. G. D. Al-Khafif et al. also uncovered evidence that, independently of social status, both high officials and workers on Giza's plain were exposed to the same threat of contracting malaria. Whereas no information on the use of protections against malaria is available for the Giza site, there is evidence that medical treatments, including surgery (i.e., limb amputation), were equally available to members of higher and lower social groups.

Some studies in this special issue had dealt with East Asian cases of ancient skeletal disease. T. Tsurumoto et al. identified a metastatic bone tumor, possibly a prostatic cancer, in a 5th-6th century Japanese skeleton. The skeleton displayed multiple osteoblastic bone lesions with a scapular sunburst appearance, cortical bone thickening with periosteal reaction, and osteosclerotic changes to the trabecular structure of cancellous bones. Y.-S. Kim et al. comprehensively analyzed lumbosacral defects and investigated the occurrence of spina bifida occulta (SBO), lumbosacral transitional vertebrae (LSTV), and spondylolysis in 16th- 18th century skeletons exhumed from Korean Joseon graves. These datasets

shed new light on the ways of living and physical conditions of ancient East Asian populations.

As highlighted in this special issue, other multidisciplinary studies also have been conducted. L. Guedes et al. diagnosed probable syphilis cases in human remains unearthed in Brazil. Historical sources had documented the occurrence of endemic treponemal infections in Rio de Janeiro between the 17th and 19th centuries. Although no morphological evidence of treponematoses was present in the analyzed samples (n = 25) obtained at the Nossa Senhora do Carmo Church (17th–19th century) and Praça XV Cemetery (18th–19th century) sites, ancient DNA analyses provided, in accordance with the historical sources, evidence of cases of probable syphilis.

Perusal of documentary sources dating to the Joseon Dynasty (17th–19th centuries) coupled with current scientific data on the evolutionary history of *P. vivax* in East Asia allowed D. H. Shin et al. to reconstruct the history of malaria in Korea. The authors highlighted how the life habits of the Joseon people might have facilitated the transmission of the protozoan, thus unwittingly contributing to the high incidence of malaria in Korea at that time.

Over the decades, mummy studies have expanded in reconstructing multifaceted databases on living conditions, pathologies, and possible causes of death in different spatiotemporal contexts. D. H. Shin et al. summarized the outcomes of studies performed in Korea and China in order to provide mummy experts with hitherto largely unknown facts on East Asian mummies. In so doing, they showed that the Korean and Chinese mummies examined share a common cultural background.

A thorough knowledge of historical backgrounds is required in order to fully assess disease burdens of past populations. The purpose of this special issue is to present new achievements in the fields of paleopathology and bioanthropology and to contextualize them in their relevant socio-cultural frames.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Dong Hoon Shin
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Robert D. Loynes
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Myeung Ju Kim

Research Article

First Paleogenetic Evidence of Probable Syphilis and Treponematoses Cases in the Brazilian Colonial Period

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Despite interest in the origins of syphilis, paleopathological analysis has not provided answers, and paleogenetic diagnosis remains a challenge. Even venereal syphilis has low infectivity which means there are few circulating bacteria for most of the individual's life. Human remains recovered from the Nossa Senhora do Carmo Church (17th to 19th centuries) and the Praça XV Cemetery (18th to 19th centuries), Rio de Janeiro, Brazil, were subjected to *Treponema* paleogenetic analysis. Historical data point to endemic treponemal infections in the city, including venereal syphilis. Based on the physiopathology of *Treponema pallidum* infection, 25 samples, mostly from skull remains of young adults, with no visible paleopathological evidence of treponematoses, were analyzed. PCR with three molecular targets, *tpp47*, *polA*, and *tpp15*, were applied. Ancient DNA *tpp15* sequences were recovered from two young adults from each archaeological site and revealed the polymorphism that characterizes *T. p.* subsp. *pallidum* in a female up to 18 years old, suggesting a probable case of syphilis infection. The results indicated that the epidemiological context and the physiopathology of the disease should be considered in syphilis paleogenetic detection. The findings of *Treponema* sp. aDNA are consistent with historical documents that describe venereal syphilis and yaws as endemic diseases in Rio de Janeiro. Data on the epidemiological characteristics of the disease and its pathophysiology offer new perspectives in paleopathology.

1. Introduction

The geographic origin of syphilis, an infectious disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* [1], is controversial. Three hypotheses have been proposed to explain the emergence of venereal syphilis. The pre-Columbian hypothesis proposes that syphilis and other human treponematoses originated in the Old World before New World contact but was misdiagnosed [2]. The Columbian hypothesis proposes that syphilis arose in the New World before the contact period and was transported to Europe by Columbus's sailors [3]. Lack of immunity against the disease facilitated rapid dispersion among Europeans. This hypothesis is supported by ethnographic reports of the use of medicinal plants for the treatment of the disease in the

New World [4, 5]. Finally, the Unitarian hypothesis suggests that there is a single treponeme with wide global distribution, and that, due to differences in climate, geographic conditions, and cultural practices, it is expressed as different forms of the disease [6]. These include syphilis and the so-called non-venereal or endemic treponematoses (ET): yaws (*T. pallidum* subsp. *pertenue*), bejel or endemic syphilis (*T. pallidum* subsp. *endemicum*), and pinta (*Treponema carateum*) [7]. All treponemes infecting humans exhibit biochemical, histological, microbiological, genetic, and antigenic similarities [4, 8]. Differences in incidence, geographical distribution, age at acquisition, main mode of transmission, clinical manifestations, capacity for invasion, and severity of late stage disease have been demonstrated [9–11]. Therefore, genetic analysis could be useful in the differential diagnosis of

Treponema species and subspecies. Complete genomes of *T. p.* subsp. *pallidum* and *T. p.* subsp. *pertenue*, which cannot be distinguished morphologically, show 99.8% identity [12]. Phylogenetic studies indicated that syphilis seems to have emerged in the Americas since *Treponema* spp. evolutionary rates are compatible with pre-Columbian times [13] and no evidence for European strains prior to the syphilis pandemic was detected [13, 14]. However, reports of pre-Columbian venereal syphilis outside the Americas and the possibility that ancient syphilis strains existed in the Old World [13, 14] imply that the topic of syphilis origin is unsolved.

Paleopathological differentiation of treponematoses is challenging. The osteological lesions caused by *Treponema* sp. and other infectious disease parasites are similar, and treponeme lesions are most pronounced in advanced stages [15, 16]. However, reproducible osseous patterns, including frequency in bone involvement, that support discrimination among treponematoses have been documented [17–19]. Ancient DNA (aDNA) analysis can be a useful tool for study of *T. pallidum* in archaeological samples and provides an opportunity to research the origins of syphilis. Kolman et al. [20] reported the detection of *T. p.* subsp. *pallidum* aDNA in a 200-year-old skeleton from Easter Island with a lesion typical of syphilis. This confirmed the first paleogenetic identification of venereal syphilis based on the polymorphism of the *tpp15* lipoprotein gene that is specific to the subspecies. Montiel et al. [21] reported recovery of *T. p.* subsp. *pallidum* aDNA from bones of human neonates recovered from the crypt of La Ermita de la Soledad (16th and 17th centuries), Spain. The aDNA was detected by PCR using *tpp15* and *arp* genes as molecular targets. Recently, Schuenemann et al. [22] used next-generation sequencing (NGS) for reconstructing genomes of *T. p.* subsp. *pallidum* and *T. p.* subsp. *pertenue* from skeletons recovered from Mexico City (17th and 19th centuries), which belonged to a perinate and infants with treponematoses manifestations.

Venereal syphilis is the most severe treponematoses, affecting various tissues and organs, including the nervous system and causing severe disability and death. It develops in three stages, and is characterized by long latent and low-infectious periods. The tertiary phase of the disease, when the typical gummatous and inflammatory bone lesions occur, is the least infectious phase. This might suggest that paleogenetic analysis could be fruitless in the study and diagnosis of treponematoses. The secondary but highly infectious phase of the disease could provide a diagnostic opportunity, although it cannot normally be identified in the osteoarchaeological record, since it is not associated with visible skeletal lesions [23, 24].

During endemic and epidemic periods, when a significant portion of the population is affected, the possibility of obtaining positive aDNA results for treponematoses by surveying skeletal keeps increases. In this study, human remains recovered from the Nossa Senhora do Carmo Church (17th to 19th centuries) and the Praça XV cemetery (18th and to 19th centuries), located in the city of Rio de Janeiro, Brazil, were submitted to *Treponema* aDNA analysis. Historical data point to endemic treponemal infections, including venereal syphilis, in the city. The investigation of bone samples from

all available individuals provided the first paleoepidemiologic scenario from the Brazilian Colonial Period.

2. Materials and Methods

2.1. Ethics Statement. This study was submitted to, and approved by, the Research Ethics Committee Nacional School of Health Public (Escola Nacional de Saúde Pública-ENSP) [AMII] (CEP number 12/2013). The bone materials are the property of the paleogenetic collection of the Laboratório de Biologia de Tripanosomatídeos (LABTRIP/IOC/Fiocruz) under the supervision of Dra. Alena Mayo Iñiguez, in collaboration with the Institute of Brazilian Archaeology (Instituto de Arqueologia Brasileira-IAB).

2.2. Nossa Senhora do Carmo Church Site. The Nossa Senhora do Carmo Church (INSC), or Old Cathedral, is located in Rio de Janeiro city, Brazil. The church became the royal chapel upon the arrival of the Portuguese royal family in 1808. In the same year, it was designated the cathedral of the city and remained so until 1976. In 2007, as part of the celebration of the bicentennial of the arrival of the Portuguese royal family, the chapel was restored [25]. During the work, several burials were discovered under the church floor, comprising Christian interments that took place until the mid-19 century [26]. The archaeological excavation was conducted by the IAB in collaboration with researchers of the Oswaldo Cruz Foundation (Fiocruz), Brazil. Articles relating to Catholicism (crucifixes) were found near or associated with the bodies buried in the nave area, and a few objects of African culture were found in the chapel area [25]. Bioanthropological analysis of skeletons *in situ* and paleogenetic collection of human remains were conducted by an anthropologist and a geneticist, respectively (Figure 1(a)). Bioanthropological analysis ($n = 32$) showed 13 of individuals were adults <35 years, 6 were mature adults >35 years, 5 were young <20 years, and 2 were children <10 years (IAB and PL/LABTRIP) [27]. The age was undetermined in the remaining 6. Eight individuals were identified as male and eight as female. Sex was undetermined in the remaining 16. The human matrilineal ancestry determined in 23/32 of the INSC individuals demonstrated that European ancestry was predominant (21/23) [27].

Paleogenetic collection involved recovering the archaeological remains in such a way as to avoid contamination during excavation as well as cross-contamination with modern DNA [28] (Figure 1(a)). It also prevented the degradation of aDNA from the time that remains were removed from their microenvironment at the archaeological site until the aDNA procedures were carried out at PL/LABTRIP [28, 29]. Measures taken during excavation included wearing protective clothing, gloves, head covering, masks, and use of sterile instruments [28, 30] (Figure 1(a)). Samples were collected in low intensity lighting and stored at 4°C protected from light in sterile containers. They were transported to the laboratory at 4°C and held at –20°C until paleogenetic analysis was performed [28].

Samples from burial, 31 comprising 14 skull fragments and 1 tooth (15 individuals), were selected for investigation. Bioanthropological data available from this study is



FIGURE 1: Paleogenetic collection, human remains, and cultural artifacts found in the Rio de Janeiro archaeological sites. (a) Procedure of paleogenetic collection of osteological samples in the INSC archaeological site performed by IAB archeologist, wearing individual protective equipment to preserve the integrity of archaeological remains; (b) a human skull from CPXV archaeological site; (c) teeth from CPXV individual, including intentional dental modifications; and (d) artifacts of African culture from CPXV archaeological site.

in Table 1. Based on the physiopathology of *Treponema* infection, the criteria for sample selection were that they should be taken from young adults [31] and show no evidence of treponematoses, in the osteological material available. Lesions compatible with treponematoses considered in this study were extensive periostitis (thickened or spiculated), of symmetrical-type, mainly in the diaphysis of the lower limbs, especially in the tibia. We also searched for localized porosities in the skulls, associated or not with hypertrophic and confluent bone reactions, as *caries sicca* type, following [9, 17, 18]. These criteria were based on the following assumptions: (1) the clinical progress of the infection is such that the highly infective secondary phase of syphilis generally occurs in young adults, increasing the probability of detectable DNA, since the number of circulating spirochetes is high only in this phase of the disease [31] and (2) when the typical bone lesions are visible during the tertiary phase, the bacterial load is low. The lack of bone lesions increases the chance of DNA recovery [15]. Skull tissue was used, as another criterion of selection, based on ready availability and its tropism for *Treponema* sp. infection [32]. In the absence of the skull in one individual, a tooth was chosen as an alternate source of well-preserved genetic material [33]. Three subadults <18 years and 3 subadults <12 years buried on burial 31, which most were young adults (n=9), were also included in the analysis (Table 1).

2.3. Praça XV Cemetery Site. The Praça XV Cemetery (CPXV) was discovered and excavated, in 1996 during the construction of a tunnel at the port of Rio de Janeiro. The cemetery was known to have received bodies of the general population who died in major epidemics with an important African slave component [34].

Thirty-seven human skeletons were recovered from secondary burials dated from 18th century. During excavation, conducted by the IAB, complete skeletal series were not identified due to a high degree of anatomic disarticulation of individuals; instead, a series of types of bones (skulls (Figure 1(b)) and mandibles, for example) were collected [35]. After excavation, samples were kept at room temperature and protected from light and underwent a curation process of brushing the surface to remove soil without use of chemicals. According to bioanthropological analysis, (n=37) 22 of CPXV individuals were young adults <30 years, 10 mature adults >40 years, 4 adolescents <17 years, and 1 child <10 years (IAB and PL/LABTRIP). Seventeen were male and 14 female, and in 6 sex was undetermined [34]. Nine of twelve individuals from which tooth samples were available showed dental modifications (Figure 1(c)) consistent with African ethnic practices but also described in Amerindian groups [36]. Objects of African culture were found around the site, as well (Figure 1(d)) (IAB and PL/LABTRIP). African, European, and Amerindian haplogroups were verified in

TABLE 1: Bioanthropological data of individuals of the present study.

Sample	Age group	Sex
INSC1a	Young adult	Female
INSC2a	Young adult	Female
INSC3a	Young adult	Female
INSC4a	12-15 years	Undetermined
INSC5a	30 years	Male
INSC6a	<18 years	Female
INSC7a	18-20 years	Female
INSC8 [*]	<25 years	Female
INSC10a	<25 years	Female
INSC11	18-25 years	Female
INSC12a	10-12 years	Undetermined
INSC13a	Child	Undetermined
INSC16	18-20 years	Female
INSC17	<18 years	Undetermined
INSC18	10-12 years	Undetermined
CPXV6a	15-20 years	Undetermined
CPXV8a	<17 years	Female
CPXV9b	<15 years	Undetermined
CPXV10a	25-30 years	Male
CPXV12a	23-25 years	Female
CPXV15b	25 years	Male
CPXV16a	25-30 years	Male
CPXV17a	25 years	Male
CPXV20a	22-23 years	Male
CPXV28a	25-35 years	Male

* A tooth was used in the absence of the skull tissue. Child: <12years; Young adult: <30 years following IAB protocol.

25% of CPXV individuals [37]. For the present study, skull fragments, corresponding to 10 individuals, were obtained from the IAB, based on the selection criteria of the study and following the procedures described above. No evidence of treponematoses was observed in the osteological material available. Bioanthropological data available from this study is in Table 1. Eight individuals were young adults <30 years and 2 subadults <17 years.

2.4. Maintaining DNA Integrity. Rigorous precautions were taken to prevent aDNA degradation and contamination by modern DNA during collection and analysis at PL/LABTRIP where sample preparation, aDNA extraction, PCR, positive PCR controls, and post-PCR procedures including electrophoresis and sequencing were conducted at the Central Laboratory (LABTRIP/IOC/Fiocruz), which is located 500m from PL/LABTRIP [28]. The replication of experiments, including PCR, electrophoresis, and sequencing was temporally separated at the Central Laboratory (LABTRIP/IOC/Fiocruz). Extraction blank controls were processed in parallel with samples, and PCR negative controls were always included. The authenticity criteria consist of the absence of detectable PCR products (pPCR) in the sediment removed from area of the sacrum (archaeological site controls),

extraction blank, and PCR negative controls. PCR positive controls were not present in the Paleogenetic Laboratory.

2.5. Ancient DNA Extraction. Prior to aDNA extraction, samples were decontaminated: the surface of the samples were removed and cleaned with 3% sodium hypochlorite. Subsequently, surfaces were irradiated with 254 nm ultraviolet light from a distance of 15 cm for 15 minutes [29]. After ultraviolet irradiation, bones were ground in an analytical mill using liquid nitrogen. Approximately 200 mg of bone powder was used for DNA extraction with proteinase K digestion as described by Iñiguez et al. [29]. Sediments (200mg) from the area of the sacrum of individuals from both INSC and CPXV, used as site environmental controls, were also submitted to aDNA extraction. For aDNA extraction, one blank control was included every six samples. The DNA IQ™ System (Promega) was used according to the manufacturer's bone extraction protocol with the modification of incubation at 56°C for 2 hours and the addition of 20 µl of 0.2M EDTA and 10 µl of proteinase K (20mg/ml) with gentle agitation. Alternatively, the QIAamp DNA Investigator-Qiagen kit was used according to manufacturer directions, with modifications: instead of the proteinase K solution specified by the kit, 30 µl of proteinase K (Invitrogen) at 20 mg/ml was used; the incubation with TAE time was increased to 10 minutes at room temperature; and final centrifugation was at 17,000 x g for 2 minutes. The concentrations of aDNA were estimated at 260 nm absorbance on a Pico200 spectrophotometer (Picodrop™).

2.6. PCR and Sequencing. Three molecular targets were applied corresponding to the *tpp47*, *polA*, and *tpp15* genes of *T. pallidum* using primers and PCR conditions according to description of each genetic marker [20, 38, 39]. The targets allowed diagnosis of syphilis and ETs but did not discriminate among *Treponema* species and subspecies, with the exception of the *tpp15* target, which discriminates venereal syphilis from ET [20]. PCR was performed in a total volume of 25 µL, using Platinum *Taq* polymerase (Invitrogen, USA) in an Eppendorf Mastercycler® PCR Cyclyer (Eppendorf, Germany). The pPCRs were analyzed by electrophoresis in 1.5-2.0% low-melt agarose gels (Sigma, USA) and visualized under UV light, after staining with GelRed Nucleic Acid Gel Stain (Biotium, USA). All positive pPCR including those of unexpected length were submitted to nucleotide sequencing. The pPCRs were directly sequenced using BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in a 3100 Automated DNA Sequencer as recommended by the suppliers. Pairwise/Blast/NCBI and BioEdit v. 7.0.1 software were used for sequence analysis. Target sequences obtained were submitted to GenBank under accession numbers KU892169-70.

3. Results

One individual from INSC (ISNC6A) and one from CPXV (CPXV8A) were PCR positive for the *tpp15* target. The remaining samples and controls, including aDNA extraction

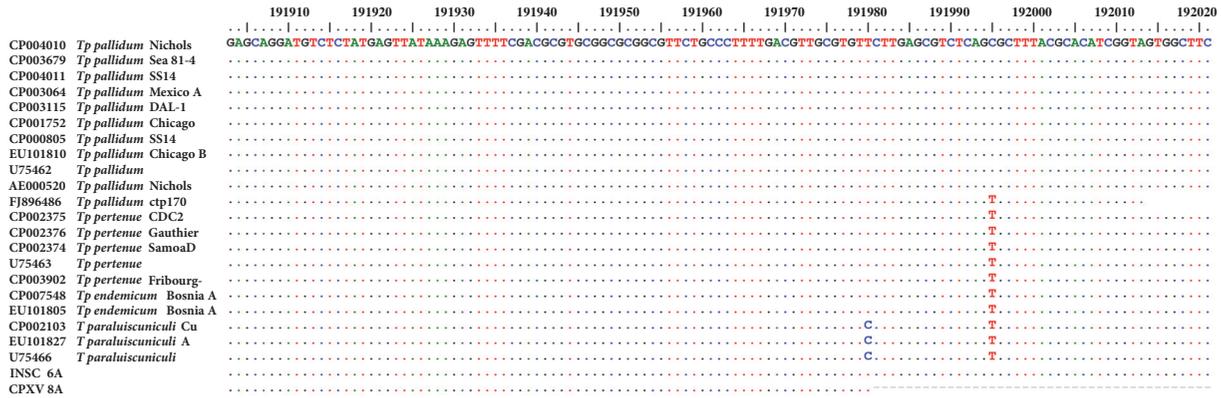


FIGURE 2: Alignment of treponemal *tpp15* target sequences. Treponemal sequences recovered in this study compared to reference sequences available from GenBank.

and PCR blanks and control sediments from the archaeological sites, were negative or presented nonspecific amplification. Nonspecific amplifications were sequenced, but no- or poor-quality sequences were obtained. Sequencing analysis of the individual from INSC when compared with reference sequence *T. p. pallidum* Nichols (Genbank CP004010) revealed the polymorphism T19195C that characterizes *T. p.* subsp. *pallidum* (Figure 2). The INSC individual with possible syphilis infection corresponded to a female up to 18 years old (Table 1). The individual from CPXV showed a short fragment in the *tpp15* conserved region that confirmed *T. pallidum* and excluded *Treponema paraluiscuniculi* (T191980C) but did not allow *T. p.* subsp. *pallidum* discrimination. The CPXV individual probably affected by treponematoses was a female up to 17 years old (Table 1).

4. Discussion

Studies have shown the difficulty in recovering aDNA of *T. pallidum*. However, Kolman et al. [20] and Montiel et al. [21] were successful in using PCR to obtain genetic material of the bacterium using spermidine, a compound that stabilizes cell membranes and DNA structure [40]. Montiel et al. [21] reported that the primary factor in their successful *T. pallidum* aDNA detection was the focus on neonate remains with evidence of CS. According to the authors, the potential for aDNA detection is higher in young individuals, due to the greater number of spirochetes distributed throughout the skeleton. Schuenemann et al. [22] also attributed the potential successfully recovering bacteria aDNA when they focused the study on young individuals. These are the only studies that have been successful in PCR recovering *T. pallidum* genetic material. Bouwman and Brown [31] evaluated the presence of *T. p.* subsp. *pallidum* and *Mycobacterium tuberculosis* aDNA in 46 human bones, some showing evidence of treponematoses and tuberculosis. Analysis of bone samples collected from an English cemetery used from the 9th through the 19th centuries did not reveal *Treponema* sp. aDNA, while *M. tuberculosis* aDNA was obtained. The authors proposed differential aDNA preservation of the pathogens. *Mycobacterium tuberculosis* is the most commonly recovered pathogen in paleogenetic analysis, due to its high load in remodeled bone

as well as the protection against DNA degradation provided by the thick and lipid rich bacterial cell membrane [31]. The lipid poor cell membrane in treponemes leaves the DNA vulnerable to degradation. Barnes and Thomas [41] analyzed *M. tuberculosis* and *T. pallidum* infections in human bones from museum collections dating from the 18th and 20th centuries. The authors concluded that an additional source of negative results in recovery of *T. pallidum* is its weak cell wall. Von Hunnius et al. [42] pointed to the lack of lipopolysaccharides, which are known to act as physical barriers to lysis, in the fragile outer cell membrane of *T. pallidum*. Spilgeman et al. [43] also discussed that the Gram-negative bacterium has high sensibility to temperature changes and easily lyses. In this study, we attributed the success of the aDNA *Treponema* detection to criteria adopted based on epidemiological data and mainly the pathophysiology of the disease.

Besides the age of remains, the type of soil, high temperatures, inadequacy on transportation, and stock conditions of sample after excavation are important factors that act in the aDNA preservation [43–45]. In this study, paleogenetic collection was applied on INSC excavation and samples were protected from the light and transported/preserved in low temperatures [27]. However, CPXV samples were obtained from IAB collection, where they were maintained at room temperature [34]. Both sites are localized in Rio de Janeiro with a tropical climate that interferes in the samples preservation [27, 34, 35, 46]. All these elements could explain negative results in most samples, but noninfected individuals cannot be ruled out.

The hypothesis of an American origin for both yaws and syphilis has been considered in many opportunities [13]. Pre-Columbian data of Brazil from Okumura et al. [47] and Eggers et al. [48] suggest treponematoses in coastal prehistoric groups dating from 2890 ± 55 to 2186 ± 60 BP and 5800–4500 BP, respectively. Filippini [49] observed bone paleopathological evidences suggestive of syphilis and yaws in Brazilian coastal sites 2000–5000 BP. In many prehistoric North American sites, bone lesions confirming yaws and even suggesting venereal and congenital syphilis have been described [50–52] but urban and historical context in the present study must be considered.

In the present work, *Treponema* sp. aDNA sequences were recovered in two young females, and the polymorphism of *T. p.* subspecies *pallidum*, the causative agent of syphilis was observed in the INSC church individual. Positive molecular results are insufficient to confirm clinical and pathological conditions, but in the positive individuals, they strongly suggest active and infective treponematoses. Studies showed similar frequencies of disease in both groups of female and male, but varying according to age, with subadults, male, and adults as the most affected [18].

Yaws and syphilis were endemic in Rio de Janeiro during the colonial period. The former was especially associated with slaves coming from Africa [53]. Syphilis was particularly important in the colonial period and, along with yaws, was mentioned by Sigaud as a disease of Africans [53]. Yaws is expected to have affected many more people than syphilis, since infants and children were infected, while syphilis, acquired through sexual intercourse, affected a different age group. Children with CS rarely survived.

Herein paleogenetic results for treponematoses reported point to possible cases of both diseases, and the epidemiological and the historical context support the findings. Poor sanitary conditions contributed to the risk of treponematoses among African slaves, which predominated among the burials of the CPXV. One individual from CPXV revealed a *Treponema* sp. aDNA sequence that, based on historical and epidemiological records, could be consistent with either yaws or syphilis. As an endemic nonvenereal disease of tropical worldwide distribution, introduced in Brazil by African slaves, yaws is most probable, but syphilis should also be pondered. Since the CPXV archaeological site has a strong African component and African cultural evidence was verified in the INSC site, a high proportion of those individuals suffering from yaws, especially those of African ethnicity, is highly probable. However, it should be considered that there is evidence for all treponemal diseases in the South America since Pre-Columbian times [47, 54, 55].

According to Edler [56], yaws and syphilis were prevalent diseases among Europeans, Native Americans, and Africans in the Brazilian imperial period. The author states that health conditions of African people were deplorable and cites contemporary depictions that illustrate this scenario, including slaves suffering from yaws. Sigaud [53] reported that yaws, to which he refers as “piã,” was spread along the Rio de Janeiro coast in the 18th century, along with other deadly diseases. According to the author, these diseases were an inevitable consequence of the slave trade that established the exchange of lethal diseases between continents.

On the other hand, it is known that syphilis became epidemic during the Brazilian colonial period [57, 58]. Araújo [59] stated that high incidence of syphilis in Brazil, especially in Rio de Janeiro, has been reported by reliable sources since the 18th century. A survey by the government of Rio de Janeiro in 1798 revealed venereal disease as epidemic in the city [37]. In the 1860s, syphilis, tuberculosis, intestinal disorders, and intermittent fevers appeared in the annual reports of health officials of the empire as diseases disproportionately affecting the poorest. According to Araújo [59], high rates of mortality from syphilis have existed since the

18th century. In 1909, Souza [60] speculated that statistics on the incidence of syphilis could be dispensed with, since the rate of those affected was similar to the total population. Other authors have reported high mortality rates from syphilis during the Brazilian historical period [58, 61]. The possible syphilis-positive individual from the Nossa Senhora do Carmo Church site, an important church for the elite of the colonial/empire period, is consistent with historical data. Further investigational PCR assays to distinguish nonvenereal *Treponema* subspecies, using new diagnostic targets and/or methodologies [62] that could be applied in paleogenetic studies, are needed for a more comprehensive panorama of treponematoses in the Brazilian Colonial period. The paucity of molecular studies on *Treponema* spp. has been contrasted by NSG studies. Recent NGS data showed treponemal genomes recovery from asymptomatic bones and PCR negative samples [63]. In addition, NGS analysis of worldwide strains showed two genomic clusters, with the emergence of syphilis strains placed in the 18th century (mean calendar year 1733, 1588-1848) and the present-day epidemic clade, in the second half of 20th century [14]. The first application of NGS for treponematoses diagnosis in archaeological samples was recently demonstrated [22]. NGS technologies open up new possibilities on syphilis paleogenetic studies to better understand the paleoepidemiology of the disease.

This study reports the first aDNA detection of *Treponema pallidum* subsp. *pallidum* that indicates a possible case of syphilis in the Colonial Brazil. Distinct populations were included in the research. Since the INSC constituted mainly Europeans of the ecclesiastical class, and the CPXV included the general population, mainly Africans slaves, it seems that the vulnerability to treponemal infection was not limited to any social or cultural group. We suggest that systematic surveys of skeletal series considering paleoepidemiological modeling could help elucidate the origins and history of treponematoses, including syphilis, in the New World.

5. Conclusions

In this study, *Treponema* sp. aDNA sequences were recovered for the first time in individuals from the Brazilian Colonial period. The paleogenetic results that suggest *Treponema* sp. infection are consistent with historical documents describing venereal syphilis, as well as yaws, as endemic diseases in Rio de Janeiro. The findings in two young females indicated that not only the epidemiological context but also the physiopathology of the disease should be considered in syphilis paleogenetic studies.

Data Availability

The nucleotide sequence data used to support the findings of this study have been deposited in the GenBank repository (KU892169-70).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Review Article

Mummification in Korea and China: Mawangdui, Song, Ming and Joseon Dynasty Mummies

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Over the decades, mummy studies have expanded to reconstruct a multifaceted knowledge about the ancient populations' living conditions, pathologies, and possible cause of death in different spatiotemporal contexts. Mainly due to linguistic barriers, however, the international knowledge of East Asian mummies has remained sketchy until recently. We thus analyse and summarize the outcomes of the studies so far performed in Korea and China in order to provide mummy experts with little-known data on East Asian mummies. In this report, similarities and differences in the mummification processes and funerary rituals in Korea and China are highlighted. Although the historical periods, the region of excavation, and the structures of the graves differ, the cultural aspects, the mechanisms of mummification, and biological evidence appear to be essentially similar to each other. Independently from the way they are called locally, the Korean and Chinese mummies belong to the same group with a shared cultural background.

1. Introduction

The dead do speak and mummies speak up. Through a comprehensive and holistic approach to the civilizations of the past, scholars aim at tracing the biological and sociocultural profiles of ancient populations back. Over the decades, the living conditions, pathologies, and possible cause of death of ancient populations in different spatiotemporal contexts (i.e., ancient Egyptians mummies, bog bodies, the Similaun Man (Oetzi), crypt mummies, the Arctic and high-altitude permafrost mummies, and South American precontact mummies) were progressively reconstructed by mummy studies [1–3].

East Asian countries have created rich cultural heritages in the continent for a long time. Over the past 60 years, several important studies were also carried out on East Asian mummies and meaningful achievements were reached. Although East Asia is not a region where a large number of mummies are found, researchers have extensively studied

these remains and have released valuable academic reports. Due to language barriers, however, most reports were not widely and efficiently diffused to English-speaking academia. From an academic point of view, scholars outside of Asia were unable to comprehensively understand the complexity of these studies.

Actually, the reality of the academic tradition of mummy studies in East Asia is distinct from other continents. East Asian mummies are culturally and biomedically so unique that extensive dissemination of cutting-edge research is paramount. Except for short introductions and sporadic reports [2, 4, 5], however, a review containing perfect data about the East Asian mummies is still lacking. And western researchers' knowledge of East Asian mummies has remained sketchy until recently. Here we thus analyse and summarize all previous studies written in Korean, Chinese, and English so to provide mummy experts with little-known information to date.

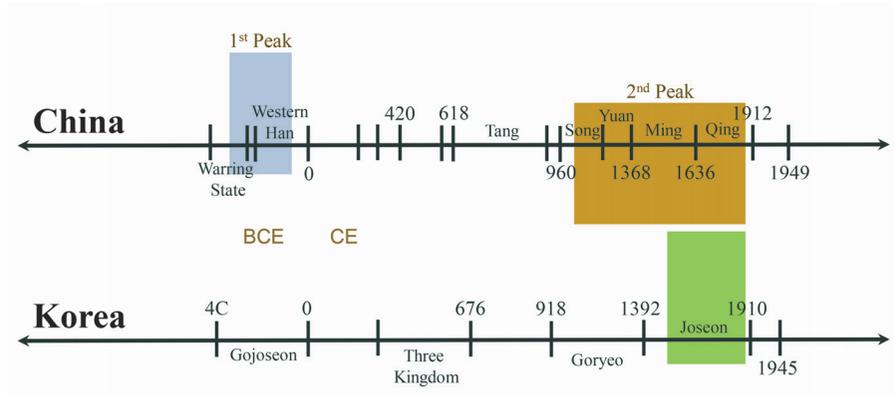


FIGURE 1: Mummies of China and Korea in the historical frame. There are two peaks in the discovery of Chinese mummies. The first peak (shaded in blue) corresponds to the Warring States (402-221 BCE) and Western Han Periods (202-8 BCE); the second peak (shaded in brown) dates to the Song (960-1279 CE) and Ming Dynasties (1368-1644 CE). The box shaded in green indicates the South Korean mummies of the Joseon Dynasty (1392-1910 CE).

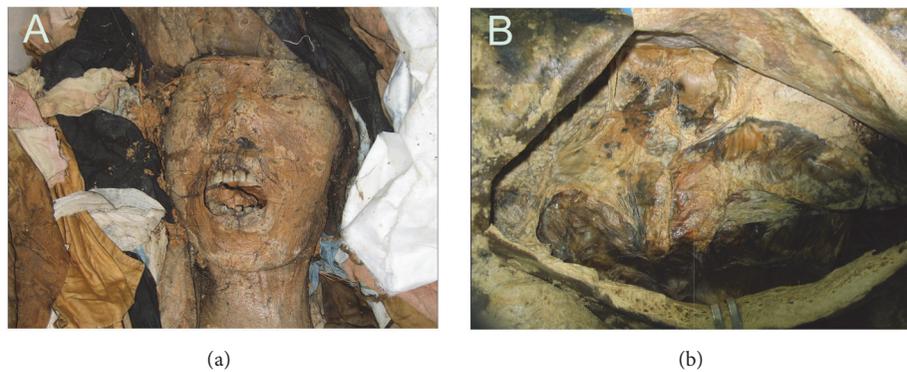


FIGURE 2: Perfectly preserved Korean mummies (Gangneung). (a) Skin and hair were intact. (b) Mummified intestines were perfectly preserved.

2. The Spatiotemporal Scope of This Review

East Asia includes the countries, districts, or municipality of Korea, Japan, China, Mongolia, Taiwan, Macau, and Hong Kong. However, this review will focus mainly on the mummies discovered in Korea and China. Here we examined the Chinese mummies of the Warring States (402-221 BCE) and Western Han Periods (202-8 BCE), those discovered in the tombs of the Song (960-1279 CE) and Ming Dynasties (1368-1644 CE) and the 16th to 18th South Korean mummies of the Joseon Dynasty (1392-1910 CE) (Figure 1). As to the ancient and excellently preserved mummies from the Tarim Basin (northwest China), their description will not be included in the present review. Given the complexity of their cultural background and antiquity (1800 BC to the first centuries BC), a separate analysis is required.

3. The 16th to 18th Century Korean Mummies of Joseon Dynasty

Over the last decade, archaeological excavations carried out in South Korea have led to uncover several 16th to 18th

century mummies buried in their original graves (Figure 1). Interdisciplinary studies [6–8] were performed on mummified tissues and organs; and precious information was gained on the lifestyle and pathologies of premodern Korean people.

Anatomical, histological, and radiological techniques were applied to verify the state of preservation of the Korean mummies (Table 1; Figure 2). According to anatomical examination, skin and hair were perfectly preserved. Histology revealed that Korean mummies' hard and soft tissues showed an excellent state of preservation. The most common histological component observed in the mummified organs was represented by collagen fibers; nevertheless, cell debris of red blood cells, chondrocytes, hepatocytes, and muscle cells were also identified. Although the brain size had shrunk, the organ still kept its original morphology. Brain tissue histology showed that myelin remnants were mainly preserved components [9–12]. Lim et al. [13] found that autofluorescence was emitted from cell residues preserved in some tissues, a finding which was interpreted with great care. As a matter of fact, during microscopic observation, it is important to ascertain whether the immunofluorescence observed in ancient tissues is specific or not [13].

TABLE 1: Joseon *Hoegwakmyo* graves investigated by interdisciplinary research.

Mummy	Research institute concerned	Year	Discovered during	Sex	Conducted research	Preservation
Kunkook	Dankook University	2001	Archaeological excavation	Female	Am, PP, PPr, CT, aDNA	Mummy
Yongin	Gyeonggi Cultural Foundation	2006	Archaeological excavation	Female	Am, PP, PPr, CT, aDNA	Half mummified
Hadong-1	Jinju National Museum	2006	Moving a grave	Female	Am, PP, PPr, CT, aDNA	Half mummified
Gangneung	Gangneung Choi clan	2007	Moving a grave	Male	Am, PP, PPr, CT, MRI, aDNA	Mummy
SN1-2	Hangang Institute of Cultural Heritage	2007	Archaeological excavation	Male	Am, PP, PPr, CT, aDNA	Half mummified
SN PK	Hangang Institute of Cultural Heritage	2007	Archaeological excavation	Male	Am, PP, PPr, CT, aDNA	Half mummified
GJ1-2	Chungnam Institute of History and culture	2007	Archaeological excavation	Male	Am, PP, PPr, CT, aDNA	Half mummified
Seocheon	Gyeonggi Cultural Foundation	2008	Archaeological excavation	Female	Am, PP, PPr, CT, aDNA	Half mummified
Waegwan	Daedong Institute of Cultural Heritage	2008	Archaeological excavation	Male	Am, PP, PPr, CT, aDNA	Half mummified
Dangjin	Chungnam Institute of History and culture	2008	Archaeological excavation	Female	Am, PP, PPr, CT, aDNA	Half mummified
Hadong-2	Descendant of Gangneung Onyang Jung clan	2009	Moving a grave	Female	Am, PP, PPr, CT, aDNA	Half mummified
Mungyeong	Gyeongju National Research Institute of Cultural Heritage	2010	Moving a grave	Female	Am, PP, PPr, CT, aDNA	Mummy
Jinju	Dong-Seo Institute of Cultural Heritage	2010	Archaeological excavation	Male	Am, PP, PPr, aDNA	Half mummified
Sapgyo	Chungcheong Institute of Cultural Heritage	2011	Archaeological excavation	Male	Am, PP, PPr, CT, aDNA	Half mummified
Sacheon	Gyeong-Sang Cultural Heritage Research Center	2011	Archaeological excavation	Female	Am, PP, PPr, aDNA	Half mummified
Hwasung	HanBeak Institute of Cultural Heritage	2012	Archaeological excavation	Male	Am, PP, PPr, aDNA	Mummy
YG2-4	Honam Institute of Cultural Heritage	2012	Archaeological excavation	Female	Am, PP, PPr, aDNA	Half mummified
YG2-6	Honam Institute of Cultural Heritage	2012	Archaeological excavation	Female	Am, PP, PPr, aDNA	Skeleton
Andong	DongGuk Institute of Cultural Properties	2013	Archaeological excavation	Male	Am, PP, PPr, CT, aDNA	Mummy
Dalsung	Gyeong-Sang Cultural Heritage Research Center	2014	Archaeological excavation	Female	Am, PP, PPr, CT, aDNA	Mummy

TABLE 1: Continued.

Mummy	Research institute concerned	Year	Discovered during	Sex	Conducted research	Preservation
Junggye	Han Ul Research Institute of Cultural Heritage	2014	Archaeological excavation	Male	Am, PP, PPr	Skeleton
Daegu_HS	Daedong Institute of Cultural Heritage	2014	Archaeological excavation	Female	Am, PP, PPr, aDNA	Skeleton
Cheongdo	Yeongnam Institute of Cultural Properties	2015	Moving a grave	Male	Am, PP, PPr, CT, aDNA	Mummy
Yeongweol	Jungbu Institute for Archaeology	2015	Archaeological excavation	Male	Am, PP, PPr	Skeleton
Jangsung	Chungcheong Institute of Cultural Heritage	2017	Archaeological excavation	Male	Am, PP, PPr	Skeleton

Am, anthropometry; PP, paleopathology; PPr, paleoparasitology; CT, computed tomography; MRI, magnetic resonance imaging; aDNA, ancient DNA.

In Korean mummy researches, radiology showed to be a highly efficient diagnostic tool [14] that enabled researchers to establish the state of preservation of the inner organs and to estimate the patient's pathological conditions in a non-invasive way. However, the radiological approach also has its own biases. Since mummified tissues and organs underwent taphonomic changes over the centuries, it may be difficult to apply modern radiological knowledge to ancient bodies. To overcome these biases (pathology versus pseudopathology), *post factum* dissections were performed to confirm the actual pattern of the mummified organs previously observed by computed tomography (CT) [15]. Magnetic Resonance Imaging (MRI) was also applied on a hydrated Korean mummy, providing researchers with invaluable information on the state of preservation of the organs with minimal damages [16]. Lastly, endoscopy showed that the organs of the Korean mummies displayed a "vivid" appearance though Kim et al. [17] were skeptical about the real efficiency of this minimally invasive technique applied to the study of ancient bodies.

Mummies have been a valuable source of information on the diseases that plagued the ancient Korean people (Table 2). For instance, atherosclerotic cardiovascular disease was confirmed in a 17th century Korean mummy by anatomical [18] and paleogenetic techniques [19]. Kim et al. [20] identified calcified pulmonary nodules in a 350-year-old-Joseon mummified individual, thus providing scholars with the oldest evidence of ancient pulmonary tuberculosis in South Korea. Thanks to multiple biomedical techniques, congenital diaphragmatic hernia [21] and Cherubism [22] were also diagnosed in Korean mummies.

Research on ancient parasites was a particularly successful field of investigation. Since the first paleoparasitological report performed on a child mummy [23], remarkable evidence of ancient parasitism was accumulated through multiple studies. Using light and electron microscopy, Shin et al. [24] showed an excellent state of preservation of ancient parasite eggs in coprolites. To date (December 2016), the paleoparasitological studies were conducted on coprolites

from 24 Korean mummies, allowing the parasite infection prevalence of 16th to 18th century Joseon people [25, 26] to be estimated. The prevalence of soil-transmitted parasites among the 16th to 18th century Joseon mummies was estimated to be 58.3 % for *Ascaris* sp. and 83.3 % for *Trichuris* sp.; and this prevalence is quite similar to the one described in the 1971 Korean National Survey. The infection rate of soil-transmitted parasites dropped with the rapid industrialization occurred during the 1980s [26]. More specifically, concerning the Trematode species, the Joseon mummies showed very high infection rates (25 % for *Clonorchis*; 33.3 % for *Paragonimus*) whereas only 4.6 % (*Clonorchis*) and 0.09 % (*Paragonimus*) infection rates were detected in the 1971 National Survey [26]. This implies that the Trematode infection rates had already decreased way before the beginning of modernization in South Korea whereas the changing pattern of the infection rates of soil-transmitted parasites in South Korea occurred around the time of modernization [26]. Why the Trematode infection rates varied before the modernization has still to be determined.

Cases of parasitism rarely seen among clinical patients were reported in the Korean mummies. For examples, ectopic (hepatic) paragonimiasis was identified in a 17th century Korean mummy [27]. A liver mass just underneath the diaphragm was identified through CT scanning; a subsequent microscopic examination revealed the presence of multiple ancient *Paragonimus* sp. eggs inside the mass. Actually, this was the first archaeoparasitological case of liver abscess caused by ectopic paragonimiasis. Another case of ectopic paragonimiasis was also observed in 400-year-old Korean female mummy [28]. Here, *Paragonimus* eggs were detected in lung, feces, intestine, and liver samples, but not in the brain nor in pelvic-cavity-debris (Table 2). The repeated reports of ectopic paragonimiasis indicate that the disease was widespread in the Korean people during the Joseon period.

Gymnophalloides seoi infection was also a unique paleoparasitological finding. *G. seoi* eggs were detected in specimens

TABLE 2: Pathological findings observed during Korean mummy studies (until September 2018).

Mummy	Estimated date	Pathological findings
Yongin	15C-16C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>P. westermani</i>
Jinju	15C-16C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>P. westermani</i>
YG2-4	15C-16C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>P. westermani</i>
YG2-6	15C-16C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>P. westermani</i>
Andong	16C	Congenital Diaphragmatic Hernia, <i>T. trichiura</i> , <i>C. sinensis</i>
Sapgyo	16C	<i>T. trichiura</i> , <i>C. sinensis</i> , <i>M. yokogawai</i> , <i>G. seoi</i>
Hadong-1	17C	<i>C. sinensis</i> , <i>M. yokogawai</i> , <i>G. seoi</i>
Hadong-2	16C-17C	<i>P. westermani</i> (Ectopic paragonimiasis)
Dalsung	16C-17C	<i>A. lumbricoides</i> , <i>T. trichiura</i>
Junggye	16C-17C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>Taenia</i>
Cheongdo	17C	<i>H. pylori</i> , <i>A. lumbricoides</i> , <i>P. westermani</i> (Liver abscess, ectopic paragonimiasis)
Sacheon	17C	<i>M. yokogawai</i>
Gangneung	17C	Lesion in the mandible, Calcified descending aorta, <i>T. trichiura</i>
Dangjin	17C	<i>H. pylori</i> , <i>A. lumbricoides</i> , <i>E. vermicularis</i> , <i>P. westermani</i>
Mungyeong	17C	Atherosclerosis, <i>M. tuberculosis</i> , <i>T. trichiura</i> , <i>C. sinensis</i>
Waegwan	17C	<i>T. trichiura</i> , <i>C. sinensis</i>
PJ SM	17C	<i>A. lumbricoides</i> , <i>T. trichiura</i>
Seocheon	17C	<i>A. lumbricoides</i> , <i>T. trichiura</i>
Yangju	17C	Hepatitis B virus, <i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>C. sinensis</i>
SN1-2	17C-18C	<i>T. trichiura</i>
SN3-7-1	16C-17C	<i>T. trichiura</i>
SN2-19-1	18C	<i>A. lumbricoides</i> , <i>T. trichiura</i>
SN2-19-2	18C	<i>A. lumbricoides</i> , <i>T. trichiura</i>
GJ1-2	17C-18C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>P. westermani</i> , <i>S. stercoralis</i> , <i>Trichostrongylus</i> spp., <i>Taenia</i>
Hwasung	18C	<i>A. lumbricoides</i> , <i>T. trichiura</i> , <i>P. westermani</i>

The full names of pathogens: *Ascaris lumbricoides*; *Trichuris trichiura*; *Enterobius vermicularis*; *Clonorchis sinensis*; *Paragonimus westermani*; *Metagonimus yokogawai*; *Gymnophalloides seoi*; *Strongyloides stercoralis*; *Helicobacter pylori*; *Mycobacterium tuberculosis*.

from two different mummies discovered on the coastal areas of the Korean peninsula [25]. Considering that *G. seoi* is not currently endemic in the counties and that the endemic focus is confined to a restricted portion in the coastal areas of the Korean peninsula [25], we speculate that this parasitic infection was more widespread during the Joseon period.

Ancient DNA (aDNA) analysis has become an important tool for revealing the phylogenetics of pathogens and the genetic profiles of the deceased. Ancient DNAs of Hepatitis B virus, *Helicobacter pylori*, *Ascaris* sp., *Paragonimus westermani*, *Clonorchis sinensis* (*C. sinensis*), and *Trichuris trichiura* (*T. trichiura*) were sequenced [28–35]. Single Nucleotide Polymorphisms were analyzed to identify ABC11, EDAR, FGFR2, and ABO genotypes [36]. In addition, various studies such as palynological analysis [37] or forensic craniofacial reconstructions [38] were successfully performed.

4. Why Did the Korean Mummies Spontaneously Preserve?

Which kind of mummification allowed the Korean mummies to preserve? Climate in Korea is not suitable for natural mummification and, before the 20th century, the Joseon did

not resort to embalming techniques [42]. Cultural beliefs implied that the intact preservation of the ancestors' corpses was an ominous sign for the descendants. In this regard, the discovery of a series of perfectly preserved mummified bodies became a sensational topic in South Korea.

Actually, the mummification process was not likely to be induced solely by natural or artificial causes, but is more likely the result of multiple, complex and synergic mechanisms. Korean researchers interested in the actual mechanism of mummification paid attention to the unique structure of the graves (called *Hoegwakmyo* or *the grave with lime soil mixture barrier*) where the Joseon people had been laid to rest [43]. During the Joseon period, lime, red clay, and sand (called *sammul* or *lime soil mixture*) were blended together in given proportions to construct the *Hoegwakmyo* tomb. The mixture was poured around the coffin and, once hardened, it completely sealed the grave (Figure 3). Since the Korean mummies were rarely found in partially or totally destroyed *Hoegwakmyo* graves, it can be inferred that the sealing itself played a major role in promoting the mummification [44]. It was also noted that a large amount of clothing was used to fill the coffins [7] (Figure 4). The use of textiles combined with the sealing produced a shortage of oxygen inside the coffin.

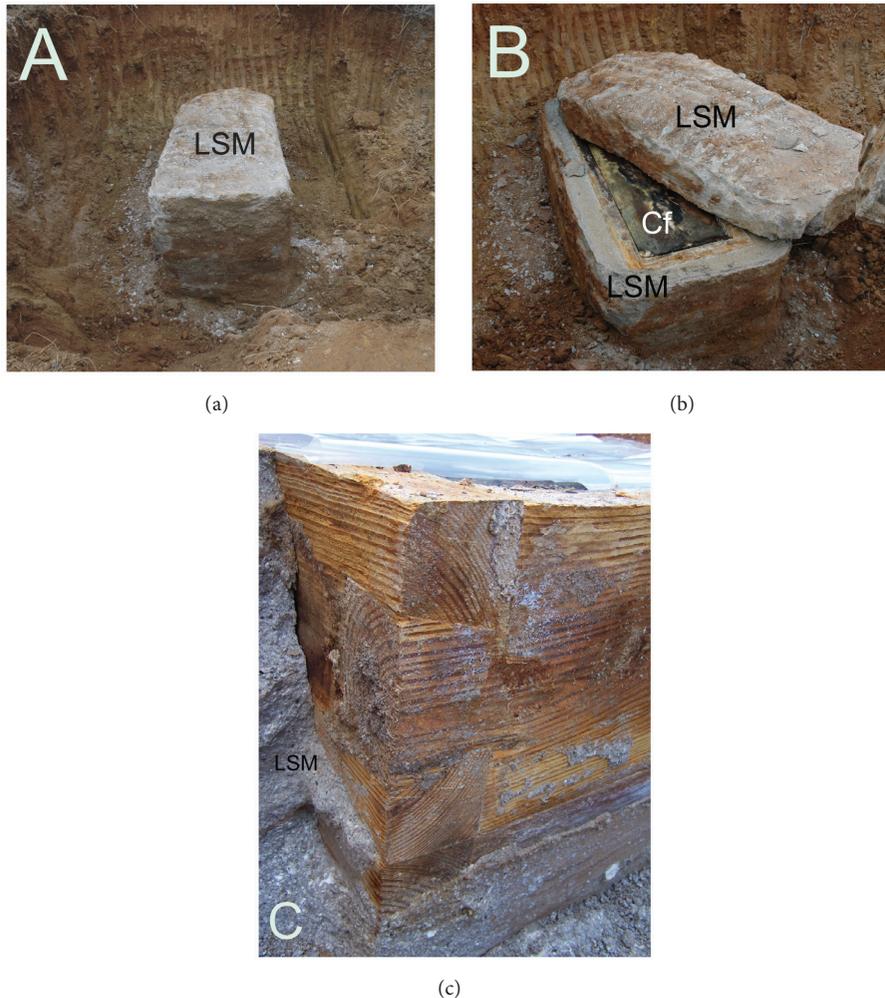


FIGURE 3: (a) and (b) An example of complete *lime soil mixture* (LSM) sealing around the coffin (Cf) of Joseon grave discovered at the Hadong archaeological site, South Korea. (c) Perfectly preserved coffin wood.

Animal experiments were carried out to reproduce the mummification process. Rats and miniature *Hoegwakmyo* grave models were used. It was observed that while there is hardening around the coffin, the *lime soil mixture* generated high temperatures. Since the heat lasted for quite a long time, it completely killed the bacteria in the animal intestines, promoting a successful mummification. Although the experiment was performed on an animal model using a miniature coffin, it is likely that a similar phenomenon occurred in the actual-sized *Hoegwakmyo* graves [42, 44]. Taken together, the use of a *lime soil mixture*, the textile filling, the low content of oxygen inside the coffin, and the high temperature produced by the hardening of *lime soil mixture* operated in synergy and enhanced the mummification process.

According to historians, the emergence of the *Hoegwakmyo* grave is closely related to the reform of the funeral rituals pursued by the Confucianists of the Joseon Dynasty (1392-1910 CE). The Confucianist ruling class people, who had overthrown the Goryeo Dynasty (918-1392 CE), believed that the funeral rituals had been seriously polluted by Buddhists'

ideals. Therefore, the funerary rituals were modified and adapted to the teachings of the Confucianism masters. The Confucianist ritual book, *Jujagare* (*Zhouziji* in Chinese), was written by the great Chinese scholar master, *Zhu Xi* (1130-1200 CE). The introduction of the book in Korea deeply influenced the funeral rites of the Joseon kingdom. In the *Jujagare*, the *Hoegwakmyo* tomb was recommended as the ideal Confucianist gentlemen's burial. The ruling class of the Joseon Dynasty assimilated this concept and the *Hoegwakmyo* tomb became their elective type of grave [5, 43]. However, what has become also evident nowadays is that the Joseon people did not want their corpses to be mummified. In that sense, the mummification in the *Hoegwakmyo* graves was a kind of unexpected accident.

5. Mummies of Sticky Rice Soup Sealed Tombs in China

Since there is a close relationship between the ancient Korean people and Confucianism, researchers hypothesized that



FIGURE 4: Clothing wrapped around the Korean mummy was found inside the Joseon grave (SN1-2).

similar tombs and mummies were present in China, the country where Confucianism originated [5]. Interestingly, Chinese archaeologists reported that some graves dating to the Song and Ming dynasties were completely sealed by a mixture of lime, yellow clay soil, sand, and sticky rice water. These tombs were called '*sticky rice paste (or soup) sealed tomb*' [5, 45]. In this regard, the Korean *Hoegwakmyo* tomb was very similar to the Chinese '*sticky rice paste (or soup) sealed tomb*' (Figures 5(a) and 5(b)).

May the *sticky rice paste (or soup) sealed tomb* be considered the prototype of the Korean *Hoegwakmyo*? According to the Chinese archaeological reports, the earliest *sticky rice paste (or soup) sealed tomb* so far discovered is the *Sun Siniangzi mu* (tomb) in the city of Jiangyin (Jiangsu province). The grave contained the mummified body of the wife of a Northern Song Dynasty (960–1127 CE) bureaucrat [5, 46]. Since the *Sun Siniangzi mu* was constructed in 1055 CE, the origin of the *sticky rice paste (or soup) sealed tomb* can be placed at the beginning-middle 11th century.

If this type of graves was related to the *Hoegwakmyo* tombs of the Joseon Dynasty, these findings would have implications also in tracing the origin of the Korean mummies back. It has to be underlined that the Chinese *sticky rice paste sealed tombs* were not identical to each other and varied in shape [5]. From this perspective, only a part of them showed similarities with the 16th to 18th century Joseon *Hoegwakmyo* graves. This implies that when the use of the *Hoegwakmyo* graves emerged for the first time in the Korean history, only a certain type of the Chinese *sticky rice paste sealed tombs* was selectively introduced in the Joseon society. Therefore, the understanding of the history of the Chinese tombs provides scholars with additional information on the history of the Korean *Hoegwakmyo* tombs.

Various biomedical studies were performed on the mummies exhumed from the *sticky rice soup sealed tombs*. In general, these corpses were very well preserved, wet-type mummies. Researchers observed that the Korean and Chinese mummies shared several common features. Both had elastic skin and flexible joints; hair, nails, and teeth showed a good state of preservation. The inner organs were fairly well preserved. These features were particularly evident in the case

of the *Xu Fan* couple found in a tomb of the Ming Dynasty [5, 40]. Histology showed intact, well-defined cartilage cells and nuclei in the mummified tissues of both individuals. Paleopathological investigations showed that the husband (*Xu Fan*) suffered from atherosclerosis and coronary artery disease [5, 40].

Another interesting case was that of the 13th century *sticky rice paste sealed tomb* containing the mummified body of the wife of the official *Wu Chou*. The mummy belonged to the Southern Song Dynasty and was found in 1988 in the De'an County (Jiangxi province). The grave dated to 1274 CE [5, 47]. A circa 30 centimetres thick *lime soil mixture* layer was present around the coffin. At the time of discovery, the body, originally wrapped in silk, was found floating in the water rising from the floor of the coffin. The preservation of the mummy was excellent. The lady was 152 cm tall [5, 47]. In terms of quantity and quality, the scientific information gained from the studies performed on the Chinese mummies was excellent and comparable to the achievements obtained by the study of the Joseon mummies.

The most advanced part of the scientific study on the Song-and-Ming dynasty Chinese mummies is the paleoparasitological one. As early as 1956, *Ascaris* eggs were identified in a coprolite from a 450-year-old male mummy belonging to the Ming dynasty. Ancient parasite eggs of *Ascaris lumbricoides*, *Fasciolopsis buski*, *C. sinensis*, and *T. trichiura* [48, 49] were also found in mummies of the Song-and-Ming Dynasties. The overall pattern of the paleoparasitological studies looks very similar to those carried on the Korean mummies.

What kind of mummification occurred inside the *sticky rice soup sealed tombs*? Chinese archaeologists proposed that multiple factors such as the complete sealing of the coffin by *lime soil mixture* (Figure 5(b)), the constant temperature/humidity inside the coffin, and other minor factors were responsible for the excellent mummification [5, 40, 50]. Again, densely packed clothing was found inside the Chinese coffins. When clothes are filled up tightly leaving no empty space inside the coffin, bacteria are unable to proliferate and die; thus, mummification occurs [5, 45, 50]. Moisture absorbent, such as charcoal, put inside the coffins and the

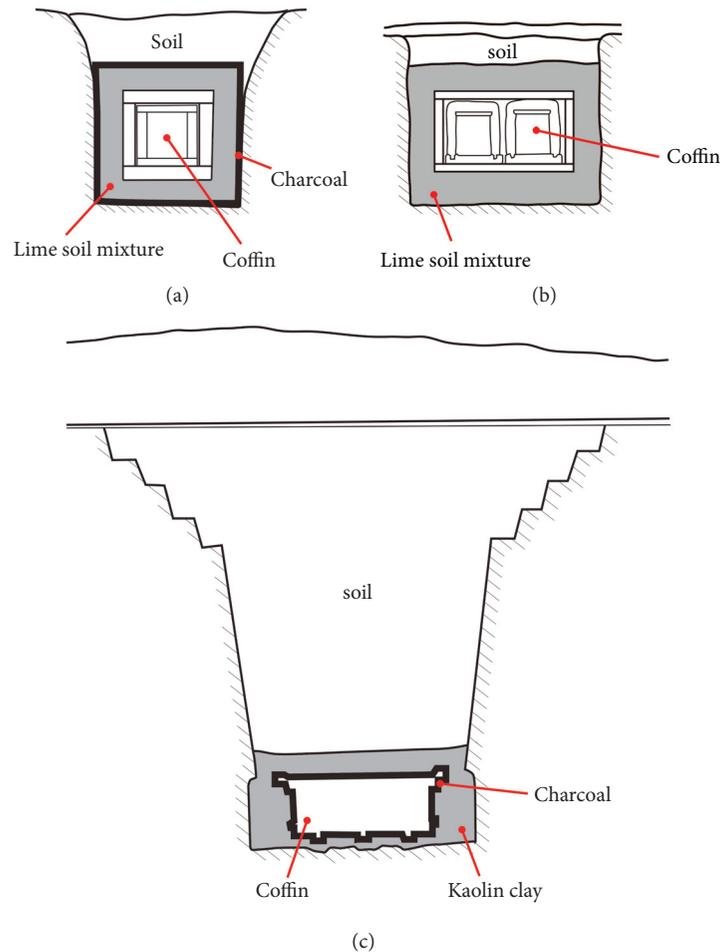


FIGURE 5: Tomb structures: (a) Korean *Hoegwakmyo* of Joseon Dynasty [39]; (b) the *sticky rice paste (or soup) sealed tomb* of Song-and-Ming Dynasty, China [40]; (c) *Mawangdui* grave of Western Han Dynasty [41]. Kaolin clay in (c) possibly played the role of sealant performed by the *lime soil mixtures* in (a) and (b). The figures herewith were redrawn based on the previous reports about *Hoegwakmyo* [39], Song-and-Ming Dynasty tomb [40], and *Mawangdui* grave [41].

bactericidal effect of lime may have been also involved in the mummification processes occurring in the *sticky rice paste sealed tombs* [5, 45, 50].

In brief, the Chinese *sticky rice paste sealed tombs* and the *Hoegwakmyo* of the Joseon society share many features, i.e., the structure of the coffin, the presence of *lime soil mixture* layer placed around the coffin, and the use of heavily packed clothing.

6. Chinese Mummies of Warring States and Western Han Period

While Korean scholars paid attention to the possible links between the mummies exhumed from the *sticky rice soup sealed tombs* and those from the Joseon *Hoegwakmyo*, Chinese scholars focused on the similarities existing among the Chinese mummies. More specifically, similarities were identified between the mummies from the *sticky rice soup sealed tombs* and those exhumed from the Warring States (402

BCE - 221 BCE) and Western Han Period (202 BCE - 8 CE) graves.

Among the Warring States and Western Han period graves so far investigated, only three corpses were classified as mummies. These corpses, which have been thoroughly studied, are currently displayed in the Hunan and Hubei provinces of China. Detailed information concerning these mummies is summarized in Table 3.

Actually, the graves of the Warring States Period (402 BCE-221 BCE) were discovered in the territory of Chu, an ancient kingdom that prospered in the present Yangtze River basin. In February 1994, grave robbers plundered a Chu tomb (*Guo-Jia Gang Tomb No. 1*) located in the city of Jingmen (Hubei province) [4, 51]. Many cultural artefacts were damaged or lost. Quite luckily, a female mummy, almost undamaged, was recovered. According to the archaeologists, the grave was constructed before Qin's unification of China (221 BCE), more precisely in the middle stage of the Warring States Period. This implies that this individual, whose corpse

TABLE 3: Mummies of Warring States and Western Han Period, China.

Mummy	Year	Excavated Sites	Date of Death (or Estimated Period)	Sex	Archaeological findings
<i>Guo-Jia Gang Tomb No. 1</i>	1994	Jingmen city/ Hubei province	Middle stage of Warring States Period	Female	Duplicated coffin (1 outer; 1 inner); Well-preserved mummy
<i>Mawangdui</i>	1971	Changsha/ Hunan Province	After 168 BCE, but not too late	Female	Multiplicated coffin (2 outer; 4 inner); Charcoal and kaolin clay around the coffin; Water on the floor of the coffin; Well-preserved mummy
<i>Phoenix Hill No. 168</i>	1975	Jiangling County/ Hubei Province	167 BCE	Male	Triplicated coffin (1 outer; 2 inner); Clay around the coffin; Water on the floor of the coffin; Well-preserved mummy

was buried in the tomb at least 2,300 years ago, represents the earliest case of mummification ever reported in East Asia to date [52].

According to the archaeoanthropological reports, a duplicated coffin (an outer and an inner coffin) was used for her burial and the coffin was found at circa 7 meters below the soil level. The state of preservation of the mummy was perfect. Aged 70-75 at death, the woman was 160 cm tall. Her blood type was AB. Parasitology revealed that she was infected by *C. sinensis* and *T. trichiura* [52]. The mummy is currently displayed in *Jingmen Museum*.

Apart from the Chu female mummy, another tomb called *Mawangdui* (*Mawangtui*) grave provided scholars with an exceptional finding. In 1971, during the construction of an air-raid shelter, a grave of the Western Han period was discovered at a depth of circa 20 meters. The archaeologists, who successfully excavated the tomb in a period of political constraints, found multiple coffins (two outer and four inner coffins) of different sizes fitted one within another. When the innermost coffin was opened, the archaeologist discovered the 'cadaver' of a woman that did not show evidence of decomposition [4]. According to archaeologists, at the time of discovery, the mummy was flooding in a liquid that filled the coffin.

The lady's name was confirmed to be *Xin Zhui*, the wife of *Li Cang* (or *Li Tsang*), Marquis of Dai (or *Tai*) during the Western Han Period. Since she died in 168 BCE, she must have been buried about 100 years later than the above-mentioned *Jingmen* mummy [4]. After two thousand years, the mummified lady and her tomb assemblage were amazingly well preserved. Researches performed on the tomb assemblage found in the *Mawangdui* grave provided scholars with valuable information about the life of this ancient Chinese lady [2].

The *Mawangdui* mummy underwent thorough biomedical investigations [53, 54]. The body of the lady, who was 154 cm tall, weighted 34.3 kg. Her blood type was A. Her skin and hair were intact, soft tissues had maintained the original elasticity, and the joints could be moved freely. X-rays showed that the skeleton was complete. At autopsy, it was shown that although the inner organs were remarkably shrunken, their relative positions had remained unaltered. Histology

showed that both peripheral nerves and skeletal muscles were well preserved [55]. Many signs of ancient diseases were identified in the *Mawangdui* lady: atherosclerosis, coronary artery disease, cholelithiasis (gallstones), lead and mercury chronic poisoning, and fracture and malunion of the distal end of the right ulna and radius. Based on the pathological evidence, it was hypothesized that the most likely cause of death was a myocardial infarction or an arrhythmia due to heart attack possibly consequent to a biliary colic [4, 56]. Muskmelon seeds (n=138.5) were found inside her intestines and paleoparasitology showed that she had suffered from *Schistosoma japonicum*, *T. trichiura*, and *Enterobius vermicularis* [49]. All these studies provided scholars with unexpected information about the life of a 2,000-year-old Chinese woman. The mummy is currently displayed in *Hunan Museum*, along with other artefacts.

Another mummy of the Western Han period was discovered in 1975. The mummy was uncovered in a Western Han Dynasty grave (*Phoenix Hill No. 168*) in the Jiangling County (Hubei Province) [57]. According to the archaeologists, a triplicated coffin (one outer and two inner coffins) was identified approximately 10 meters underneath the soil surface [4, 58]. Similar to the *Mawangdui's* Lady, the corpse was immersed in dark red fluids (100 liters at a depth of 75 cm) [4]. The body belonged to a male, named *Sui*, an official (*wutai fu* or *wutafu*) of Western Han Dynasty who had died in 167 BCE when he was ca 60 years old [4].

Anatomical and histological studies confirmed that the state of preservation of the mummy was excellent. Soft tissues had maintained their elasticity. No hair was preserved whereas all teeth were present. The body measured 167.8 cm and weighted 52.5 kg; the blood type was AB. Autopsy showed well-preserved inner organs. In general, histology showed that most cells had disappeared whereas the collagen fibers were still abundant. The main component of the nervous tissue was represented by myelin remnants [4, 59]. No osteoporotic changes were found. The man suffered of chronic cholecystitis, parasitic hepatic cirrhosis, gallstone, and atherosclerosis. The cause of death was attributed to acute peritonitis due to a chronic gastric ulcer at the lesser curvature of stomach complicated by acute perforation [2, 59, 60]. Parasitology showed the presence of ancient eggs of *S.*

japonicum, *C. sinensis*, *Taenia* sp., and *T. trichiura* [2, 49, 59, 61]. Sui's mummified body is currently displayed in Jingzhou Museum.

The 2,000-year-old mummies of the Warring States (402 BCE - 221 BCE) and Western Han period (202 BCE - 8 CE) have a high reputation in China. The perfectly preserved artefacts recovered from their graves provided scholars with information that, otherwise, would not have been obtained through the historical and archaeological studies.

The three Warring States and Western Han mummies seem to share some factors that favoured their preservation. Besides temperature, humidity, pressure, and pH, more inducing factors were suggested to be responsible for such an excellent preservation. Among these factors are the air-tight sealing of the coffin, the depth of the burial (Figure 5(c)), and the presence of cinnabar (HgS) in the liquid found inside the coffins at the time of discovery [4, 59]. It is also noteworthy that charcoal and kaolin clay (Bai gao ni) were used in sealing the coffins [56]. Kaolin clay may have played the same role of sealant performed by the *lime soil mixture* in the *Hoegwakmyo* graves and in the *sticky rice paste sealed tombs* (Figure 5). The complete sealing of the Warring States-Western Han graves by kaolin clay may have prevented the percolation of water and air into the coffin, thus creating anoxic conditions inside the inner coffin and halting the decomposition [56, 62]. The charcoal layer of the *Mawangdui* tomb may have also contributed to the preservation of both mummy and artefacts by absorbing the moisture that otherwise would have seeped inside the coffin [56].

7. Mummies of Korea and China in East Asian History

As shown in the present review, the Chinese mummies date back mainly to two historical periods. The first period corresponds to the Warring States and Western Han Periods (circa 2,000 years ago). The majority of the mummies dating to these periods were uncovered in the Hubei and Hunan Provinces. For next almost 1,000 years, very few mummies dating to the first millennium CE were found in China. When the second millennium began and the Chinese constructed the *sticky rice paste sealed tombs* during the Song and Ming dynasties, mummified bodies were newly recovered.

As previously stated, the Korean mummies unearthed from the *Hoegwakmyo* graves appear to be closely related to the Chinese mummies found in the *sticky rice paste sealed tombs*. Interestingly enough, the Chinese scholars did not consider the mummies belonging to the two distinct historical phases (Warring States and Western Han period versus Song/Ming dynasties) as separate entities. Rather, the corpses were classified as *wet corpses* [50], *ancient corpses* [4], or just *cadavers* [4, 54, 56], thus emphasizing the differences between the Chinese mummies and the mummies from other continents. In China, the so-called '*Mawangdui type cadaver*' generally includes the Warring States and Western Han Period mummies as well as the Song/Ming period mummies [4]. In our opinion, the corpses exhumed from the two different Chinese historical phases and the Korean mummies should be included in the same category.

Actually, Korean and Chinese mummies share many cultural and biological characteristics. Researchers agreed that both the mummification and the artefacts' preservation in the *Mawangdui* like graves were most likely due to the presence of a kaolin clay layer surrounding the coffin [56, 63-65]. Wang and Zhang [50] also proposed that the complete sealing of the coffin obtained resorting to the *lime soil mixture* was an important inducing factor for mummification in the *sticky rice paste sealed tombs*. In brief, the complete isolation of the inner coffin, either with kaolin clay or *lime soil mixture*, was responsible for mummification both in China and in Korea. The funerary customs also played an important role in the mummification process. For example, the tight packing of the cloths inside the coffin was a shared funerary custom in China and Korea. And above all, both in Korea and in China, the tombs' construction followed the precepts of the Confucianist tradition.

From a biomedical perspective, the mummies of Korea and China show considerable similarities. In both countries, the dead body did not undergo embalming and the internal organs were not removed from the body cavities. The macroscopical and radiological appearance of the mummified organs was similar in the Chinese and Korean examined cases. All inner organs were considerably shrunken and distorted, displaced dorsally but fixed in their relative position. Also, the histological findings were similar in mummies from both countries. In the case of the *Mawangdui* type mummies, microscopy revealed that the collagen fibers were the best preserved component. A considerable number of chondrocytes were also found in cartilages and myelin remnants were the predominant structures identified in the nervous tissue [4]. A closer look at the histology of the Korean mummies reveals a strict similarity with the Chinese *Mawangdui* type mummies.

8. Conclusions

Interdisciplinary researches performed on the Korean mummies have led to gain invaluable scientific information on the health and disease statuses of past populations. Thanks to a growing body of literature written in English, the international scientific community has rapidly recognized the distinctiveness of the Korean mummies. Conversely, except for some brief reports, the investigations performed on the Chinese mummies were seldom presented to the international academia [2, 5]. Nevertheless, since the 1970s, China had a long history of high quality research on mummified bodies. The present review contains a synthesis of the studies carried out so far on the Korean and Chinese mummies.

It is worth noting that strict cultural and biomedical similarities can be identified among the Chinese *Mawangdui* type mummies, the mummies of the Song-and-Ming dynasties (the *sticky rice soup sealed tombs*), and the Korean mummies of Joseon dynasties. Even if the historical periods, the regions of excavation, and the structures of the graves varied, similarities in the mummification processes and funerary rituals are also highlighted. In this study, this statement is also confirmed by biological evidence. Independently from the way they are locally called, the East Asian mummies belong to the same group with a common cultural background.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

An Ancient Skeleton with Multiple Osteoblastic Bone Lesions Containing a Scapular Sunburst Appearance from a 5th–6th Century Grave Excavated in Oita, Japan

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A human skeleton of a middle-aged adult male was found in a 5th–6th century Kinoue-Kodo stone coffin excavated from the southwest marginal region of the Oita plains, northeast Kyushu, Japan. The skeleton was buried respectfully in the ancient tomb, and red pigment was applied to his face after death. We report herein findings from computed tomography imaging of the skeleton and discuss the multiple osteoblastic lesions identified in the humerus, scapula, clavicle, vertebra, pelvic bones, and skull of this individual. These lesions comprised cortical bone thickening with periosteal reaction localized to the surface and osteosclerotic changes mainly observed in the trabecular structure of cancellous bone. In particular, a typical sunburst pattern was also noted on the left scapula as another characteristic lesion found in this case. By differential diagnosis, the disease suffered by this individual was most likely to be metastatic bone tumors, especially of prostate cancer. This person may have survived until many bone metastases had developed throughout his whole body.

1. Introduction

In archaic societies in which the natural and social environment surrounding human living areas and the demographic composition were markedly different from the present time, it might be thought that the incidence of cancer was lower than nowadays. However, the frequency of malignant tumors developing in young people (adolescents and young adults), such as osteosarcoma and leukemia, may not have been so different. When ancient human skeletons excavated from ruins are observed, benign tumors, such as osteomas of various sizes, are not rare, but it is very rare to discover traces of malignant tumors in a skeleton. In this study, we closely investigated multiple bone lesions noted in an adult male human skeleton buried in a 5th–6th century sarcophagus tomb of the Kofun period found in the plains of Oita prefecture, Kyushu, Japan.

2. Materials and Methods

This human skeleton was excavated from a Kinoue-Kodo stone coffin in a Kinoue-Touge stone coffin group located in the southwest marginal region of the Oita plains of northeast Kyushu (Figure 1). According to the archeological findings, this ruin was considered to have been constructed in the 5th–6th century. There was a big ancient tomb in this region that was an “ancient imperial tomb” of 80 m in length, 58 m in diameter, and 9 m in height. It is considered to be the tomb of a powerful family ruling this region in the middle of the 5th century. Kinoue-Kodo stone coffins were discovered near this imperial ancient tomb, and three box-shaped stone coffins constructed by combining plate-like broken stones with an andesite nature were aligned in a south–north direction. At least two adult human skeletons were buried in one of these coffins. We report here on the human skeleton on the

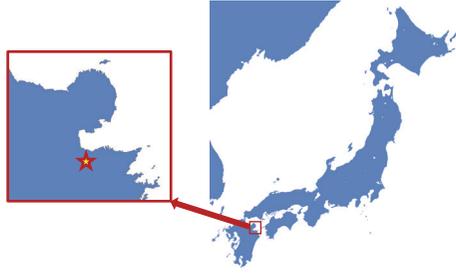


FIGURE 1: Remains of the Kinoue-Touge stone coffin group located in Oita city, Oita Prefecture, Japan.

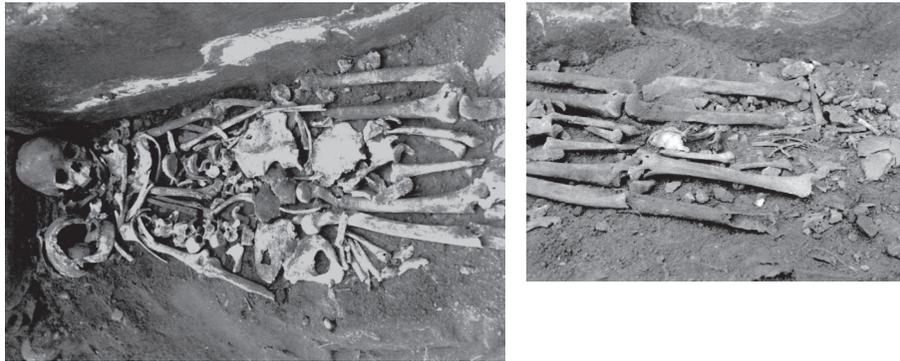


FIGURE 2: Photographs of two adult human skeletons buried in one of the Kinoue-Kodo stone coffins. The subject was the human skeleton on the left, which was considered to be a middle-aged male.

left, which was considered to be a middle-aged adult male (Figure 2). In previous report, Tashiro [1] speculated that this human skeleton in a 'Paleopathological study of skeletons excavated from Kyushu Island', in which this individual was considered to have prostate cancer before death. We report this case in detail with computed tomography (CT) images and discuss the case in relation to the current literatures. The CT images of the pathological bones in this skeleton were obtained by using clinical CT equipment (Activision 16, Toshiba Corp. Japan); the sampling interval was 0.5 mm, and the data were saved in DICOM (Digital Imaging and Communication in Medicine) format. Free DICOM Viewer 1.4.5.0 (YAKAMI Software) software was used to display them.

3. Result

3.1. Condition of the Human Skeleton. As shown in Figure 3, the skull, bilateral humeri, ulnas, radii, femora, tibiae, clavicles, scapulae, sternum, and pelvic bones were mostly or partially present. The condition of the remaining skull was favorable, and red pigment had been applied to the surface, mainly on the frontal face, after death.

3.2. Skull (Figure 4). The skull mostly remained in a relatively favorable condition excluding the right zygomatic bone, most of the left parietal bone, and part of the occipital bone. It was also clear that red pigment had been placed on the surface,

mainly on the frontal face, after death, and the superficial layer of the bone surface had come off to various extents (Figure 4(a)). Thus, macroscopic observation of some parts was difficult. First, the appearance was observed. The metopic suture remained over the entire length in the midline of the frontal bone. Bulging lesions were present on the cortical bone surface of the bilateral inferior orbital walls. A 15×15 mm bulging lesion with a slightly porous surface was present on the left superior orbital wall (Figure 4(b)). On CT images, a solid osteoblastic lesion continuing between the inner and outer cortex was noted (Figures 4(d), 4(e), and 4(f)). In addition, a 20×20 mm prominent irregular lesion was present on the outer wall surface of the right orbit, in which a spicular formation showing periosteal reaction was noted on the surface (Figure 4(c)). At present, macroscopic observation of the inner surface of the skull is not possible.

3.3. Left Scapula (Figure 5). The left scapulae, mainly the glenoid cavity region, remained partially. Of its glenoid cavity, the coracoid, lateral margin, and base of the scapular spine were continuous and remained as one bone fragment. The first finding that attracted attention was an osteoblastic lesion comprised of densely concentrating small needle-like or plate-like protruding bone expanding over in a 35×40 mm region on the posterior surface of the base of the glenoid cavity below the base of the scapular spine (Figures 5(a) and 5(b)). The angle changed in a regular manner from the central area to the marginal region, and its overall morphology

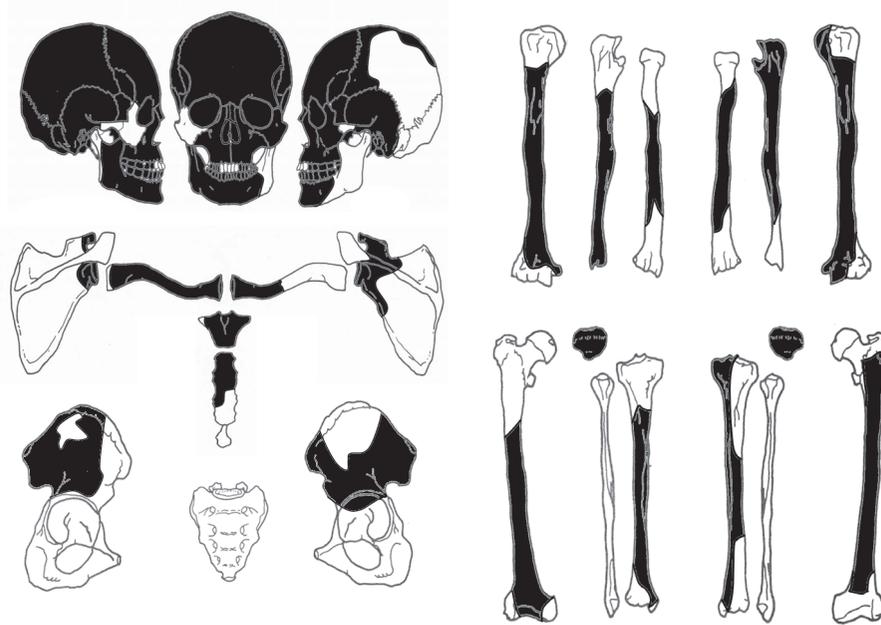


FIGURE 3: Condition of the human skeleton. The skull, bilateral humeri, ulnas, femora, tibiae, clavicles, scapulae, sternum, patella, and pelvic bones remained mostly or partially.

showed a sunburst appearance. The lesion was continuous with proliferative lesions on the bone surface at the base of the coracoid. In addition, a lesion with a sunburst appearance, although it was incomplete, was also present on the anterior surface of the left scapula, mainly at the base of the coracoid (Figure 5(d)). In CT images of the left scapula, the presence of needle-like osteoblastic lesions was confirmed expanding continuously on both the anterior and posterior surfaces at the base of the glenoid cavity (Figure 5(c)).

3.4. Right Scapula, Right Clavicle, and Vertebral Body (Figure 6). The right scapula, mainly the glenoid cavity region, remained partially (Figure 6(a)). Overall, the bone was markedly broken, but osteoblastic lesions were present extensively on the bone surface excluding the joint surface.

About one-third of the proximal region of the right clavicle remained, but this was also broken (Figure 6(b)). However, on macroscopic observation of the surface, worm eaten-like lesions were present almost circumferentially. On CT images, intermixed osteosclerosis and osteolysis were continuous from the bone surface to the inner region.

One vertebra remained, but the condition was poor and only the vertebral body remained partially (Figure 6(c)). Based on the size, it was considered to be a thoracic or lumbar vertebra, but it was unclear which vertebra it was. Cancellous bone was markedly thickened over the entire residual vertebral body, appearing to be osteosclerosis. This was also noted on CT images.

3.5. Right Humerus (Figure 7). In the right humerus, the proximal end containing the entire bone head and distal joint surface was missing. Of the remaining regions, lesions

showing irregular minute osteoblastic changes were present on the anterior surface of the proximal region and on the bone surface of the lesser tubercle over the intertubercular sulcus (Figure 7(a)). On CT images, osteosclerosis was observed continuing from the superficial layer to the inner region and in the proximal metaphysis of this humerus (Figures 7(b), 7(c), and 7(d)).

3.6. Pelvic Bones (Figure 8). The pelvic bones remained as a partial block centered on the ilium on both sides. The right coxa was present as a block containing the anterior half of the ala of the ilium and part of the acetabular roof (Figures 8(a) and 8(b)). The left coxa remained as a bone fragment containing the anterior part of the auricular surface of the ilium (Figures 8(c) and 8(d)). Because both coxae were predominantly broken, accurate macroscopic evaluation of the properties was difficult, but cortical and cancellous bones were replaced with osteoblastic lesions from the surface over the inner region across most of the remaining region; i.e., the surface was rough and irregular, showing a periosteal osteoblastic pattern, and it did not stay on the bone surface but instead continued into the deep inner region. The trabecula was markedly thickened in partially glimpsed cancellous bone. These lesions had properties similar to changes in the vertebral body described above. In CT images, osteosclerosis and osteolysis continuing from the bone surface to the inner region were mixed, similar to that noted in the vertebral body and clavicle.

In addition, the left humerus and bilateral ulnas, femora, tibiae, and patellae remained partially, but no lesions were noted on the surfaces of any bone from macroscopic observations.

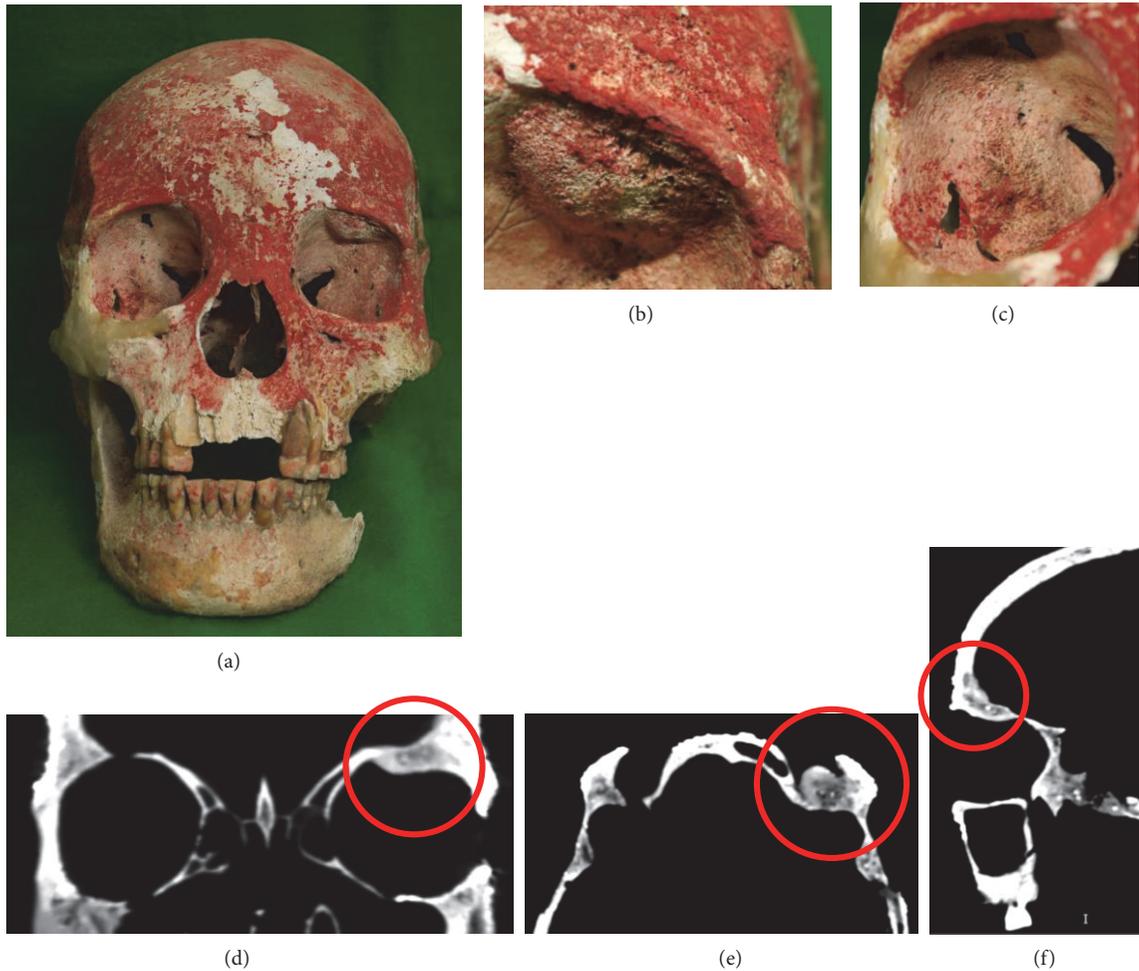


FIGURE 4: Photographs of the skull. Red pigment was applied mainly on the frontal face region after death (a). In the left superior orbital wall, a 15×15 mm bulging lesion with a slightly porous surface was present (b) in which a solid osteoblastic lesion continuous with the inner region was noted on CT images (d, e, and f). On the inner wall surface of the right orbit, a 20×20 mm irregular prominent lesion was present (c).

4. Discussion

4.1. Diseases to Be Subjected to Differential Diagnosis. The characteristics of bone lesions in this case included the presence of multiple osteoblastic lesions throughout bones from across the entire body, and these were comprised of cortical bone thickening with periosteal reaction localized to the surface and osteosclerosis mainly showing changes in the trabecular structure of cancellous bone. Based on these findings, the diseases present in this case were differentiated as described below.

A typical disease responsible for the development of osteoblastic bone tumors could be a representative primary malignant bone tumor, such as osteosarcoma. Smith-Guzman [2] reported a case of primary malignant bone tumor observed in the right humerus excavated from a Pre-Columbian era ruin from A.D. 1265–1380, Aufderheide [3] reported a case of osteosarcoma showing a typical sunburst appearance, which developed in the distal humerus and femur in an adult native Peruvian 800 years ago, and Bona [4] reported the results of an investigation of osteosarcoma

in the right humerus using a biomarker in a 25–35-year-old female excavated from an approximately 2,000-year-old ruin. All these cases developed in a solitary bone. Osteosarcoma is considered to be a highly malignant disease, and it metastasizes to visceral organs from an early stage, but the development of multiple bone lesions in distant regions is unusual. Accordingly, osteosarcoma is unlikely to be the disease in the present ancient case without any available therapy.

Another representative primary malignant bone tumor, Ewing sarcoma, shows osteolysis on radiography as a rule, such as a permeative (small holes) or moth-eaten (mottled) appearance, but these may be accompanied by periosteal reaction, expressed as lamellated, sunburst, or spiculated patterns, in proximity, and it may show Codman's triangle similar to osteosarcoma. In cases intensively treated using modern medical care, the development of several bone lesions is not rare. Therefore, although the possibility is low, it cannot be ruled out that this was a case of Ewing sarcoma.

Among metastatic bone tumors, diseases showing an osteoblastic pattern are candidates for differential diagnosis.

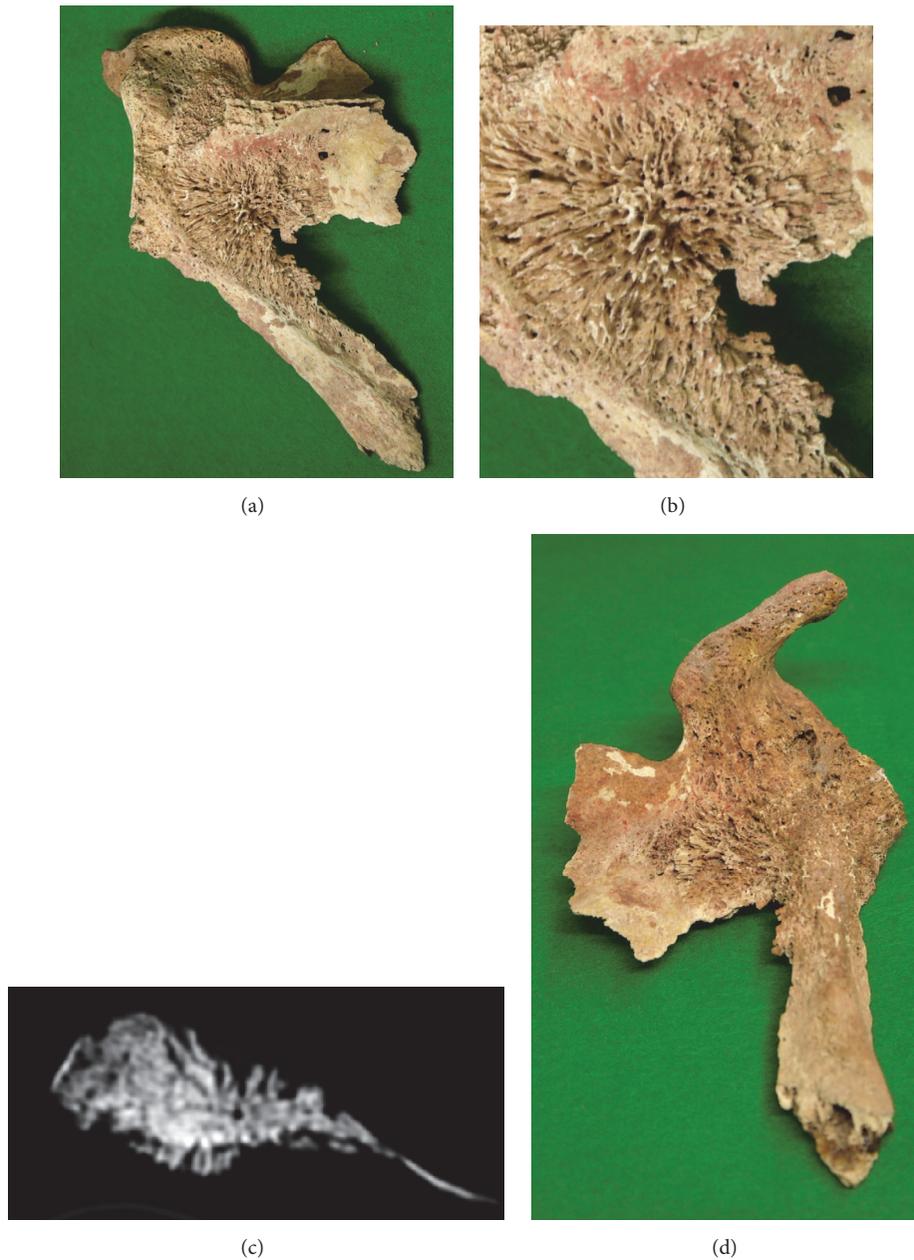


FIGURE 5: Photographs of the sunburst appearance noted on the left scapula. An osteoblastic lesion was present, comprised of small, densely concentrating, needle-like or plate-like protruding bones expanding in a 35×40 mm posterior region at the base of the glenoid cavity below the base of the scapular spine (a, b). This lesion continued to the proliferative lesion on the bone surface located at the base of the coracoid (d). On CT images, the presence of needle-like osteoblastic lesions was confirmed expanding continuously across both the anterior and posterior surfaces of the base of the glenoid cavity (c).

Klaus [5] observed osteoblastic and osteolytic lesions in the lumbar spine and sacrum in four human skeletons excavated from ancient ruins in Peru from the period A.D. 900–1600 and considered that these were cases with metastatic lesions of prostate cancer. They considered blood diseases, such as myeloma, leukemia, and Hodgkin's and non-Hodgkin's lymphomas, bone- and cartilage-derived primary malignant tumors, such as sclerosing osteosarcomas and chondrosarcomas, and benign and malignant tumors, such as

meningiomas, hemangiomas, and adrenal neuroblastomas, for differential diagnosis in the diagnostic course. It was considered that bone metastasis of prostate cancer was the most suspicious for the development of multiple osteoblastic lesions in bones throughout the whole body.

Furthermore, to infer the diagnosis of the present case, the characteristic periosteal proliferation, i.e., the sunburst pattern, observed on the dorsal surface of the left scapula may be the key. Periosteal reaction on the cortical bone surface

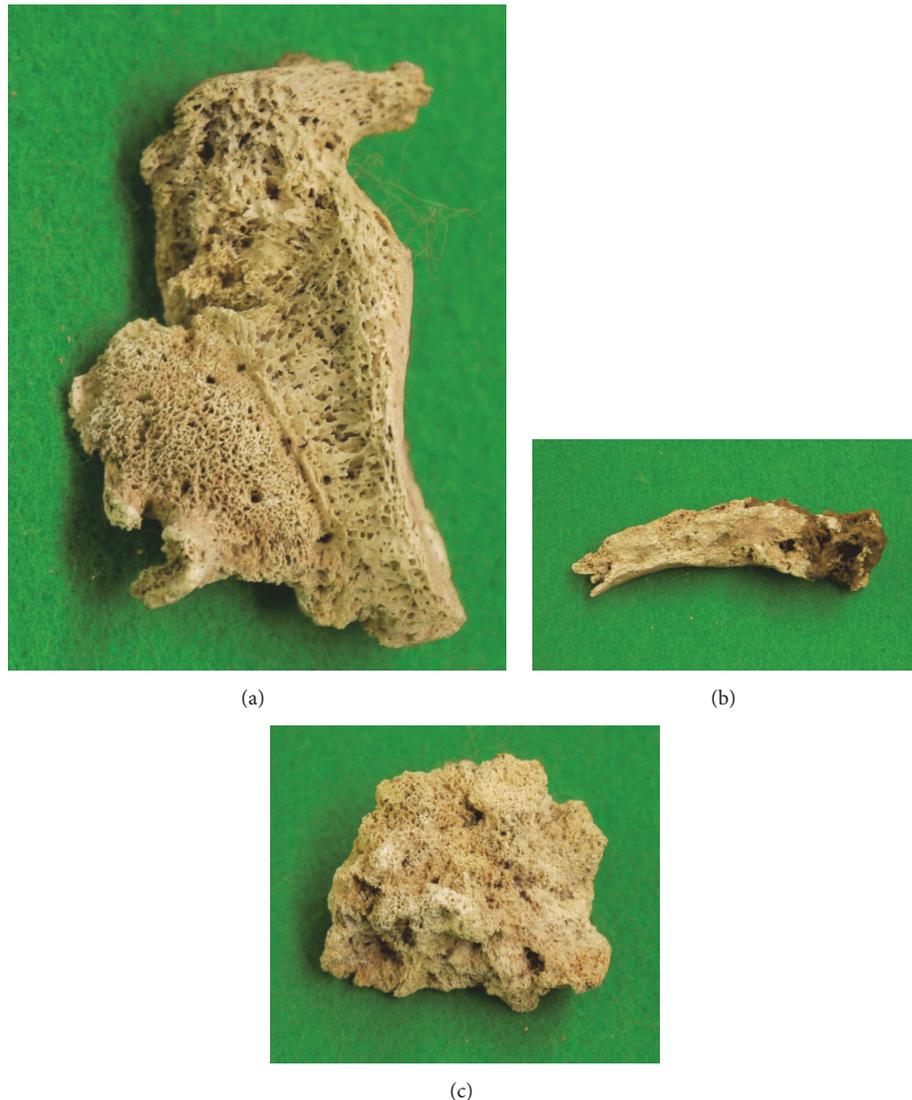


FIGURE 6: Photographs of the right scapula (a), right clavicle (b), and vertebral body (c). The right scapula, mainly the glenoid region, remained partially (a). Overall, the bone was markedly broken, but osteoblastic lesions were present extensively on the bone surface excluding the joint surface. On the right clavicle, worm eaten-like lesions were present almost circumferentially on the surface (b). On the vertebral body (c), cancellous bone was markedly thickened across its entirely, showing the features of osteosclerosis.

occurs due to elevation of the bone surface periosteum caused by various bone tumors, infection, trauma, drug administration, or arthritis, and it shows various patterns on radiography depending on the intensity, aggressiveness, and duration of the underlying insult of the lesion [6]. Of these, a sunburst pattern is observed when rapidly growing tumors develop simultaneously in bone and the surrounding tissue without destructive changes [7]. Spiculated periosteal reaction is most frequently noted in primary bone tumors, but it is also sometimes observed in metastatic bone tumors.

Vilar [8] summarized six cases of metastatic bone tumor and stated that bone metastasis should be included in differential diagnosis of perpendicular periosteal reaction at 40-years-old or older. Lehrer [7] also reported five similar cases where the primary lesion was retinoblastomas in two

cases, prostatic carcinoma in one case, tumor of undetermined primary origin (probably bronchogenic) in one case, and chloromatous acute myelocytic leukemia in one case. Bloom [9] surveyed 20 periosteal sunburst reaction cases of metastatic bone tumors, in which the most frequent primary lesion was prostate cancer. Therefore, periosteal reaction or sunburst periosteal proliferation may occur in metastatic bone tumors similar to that in primary bone tumors. In the present human skeleton, multiple osteoblastic lesions were present in bones throughout the whole body, but osteolytic lesions were almost absent in the remaining bones, and the skeleton was judged as a middle-aged male. Based on these findings, it was considered that metastatic bone tumors, such as those from prostate cancer, were the most likely lesion. Furthermore, Hove [10] initially reported two clinical cases

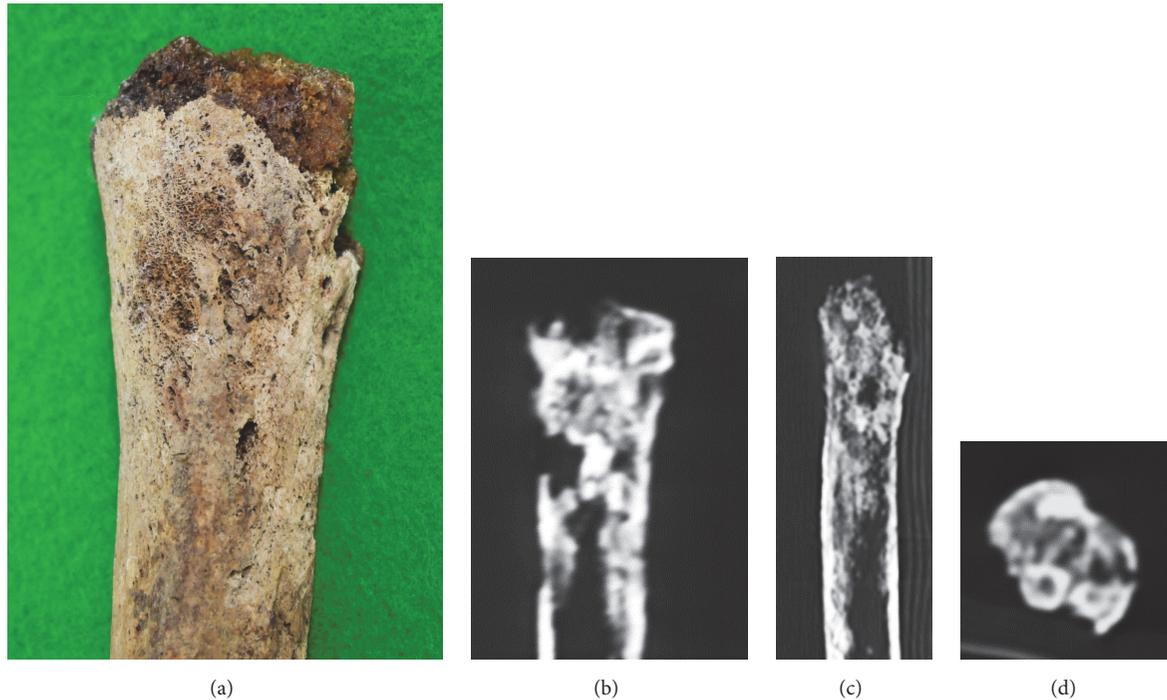


FIGURE 7: Photographs of the right humerus. Lesions showing irregular minute osteoblastic changes were observed on the anterior surface of the proximal region and bone surface of the crest of the lesser tubercle over the intertubercular sulcus (a). On CT, continuous osteosclerosis was observed from the superficial layer to the inner region in the proximal metaphysis of this humerus (b-d).

in which spicule formation was noted in metastatic vertebral body lesions of prostate cancer on CT images.

4.2. Paleopathological Report on Cases of Bone Metastasis of Prostate Cancer. In paleopathological samples, several cases of bone metastasis of prostate cancer have been reported. Wakely [11] reported findings suggestive of metastatic lesions of prostate cancer observed using radiography and scanning electron microscopy in an elderly male skeleton excavated from a medieval ruin from the 14th century in the Canterbury region of England. Schultz [12] noted rough bone-forming lesions on the iliac and costal bone surfaces in a male human skeleton from 2,700 years ago in his 40–50s excavated from South Siberia and considered that it was a case of bone metastasis of prostate cancer. Ghabili [13] reviewed 17 cases of bone metastasis of prostate cancer, including bones excavated from approximately 3,300-year-old ruins in Spain and a case excavated from a tomb in England in 1834, in which the affected bones included the pelvis, femur, spine, clavicle, skull, scapula, humerus, ulna, radius, fibula, carpal, and tarsal bones, showing extensive distribution. More than half of these were an osteogenesis/osteolysis mixture, but five cases contained only osteoblastic lesions. Osteoblastic lesions were widely distributed in the axial skeleton, such as the pelvis, scapula, costal bone, and spine, and four extremity bones, such as the humerus and femur. Of these, the clavicle of a skeleton excavated from a 19th-century tomb reported by Waldron [14] was the only case showing a “sunburst pattern” similar to that in the present case.

5. Conclusion

This skeleton with multiple metastatic bone tumors was buried respectfully in an ancient tomb and red pigment was applied to the face after death. Among the Yayoi people in the Kofun period in Japan, vermilion color was applied to the face with mercury pigment and red iron oxide as a burial ritual. In this study, red pigment was also applied to the facial bone. Archeologically, it is considered that the red color was magical and sacred and had meaning as a “talisman” or hoping for ‘resurrection of the dead’. Considering these findings, this person appears to have been buried respectfully in an ancient tomb after death. As for this case, the most likely disease was suggested to be metastatic bone tumors, probably from prostate cancer. Among malignant tumors, prostate cancer shows relatively slow progress. Therefore, this person might have survived until many bone metastases developed throughout the whole body.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

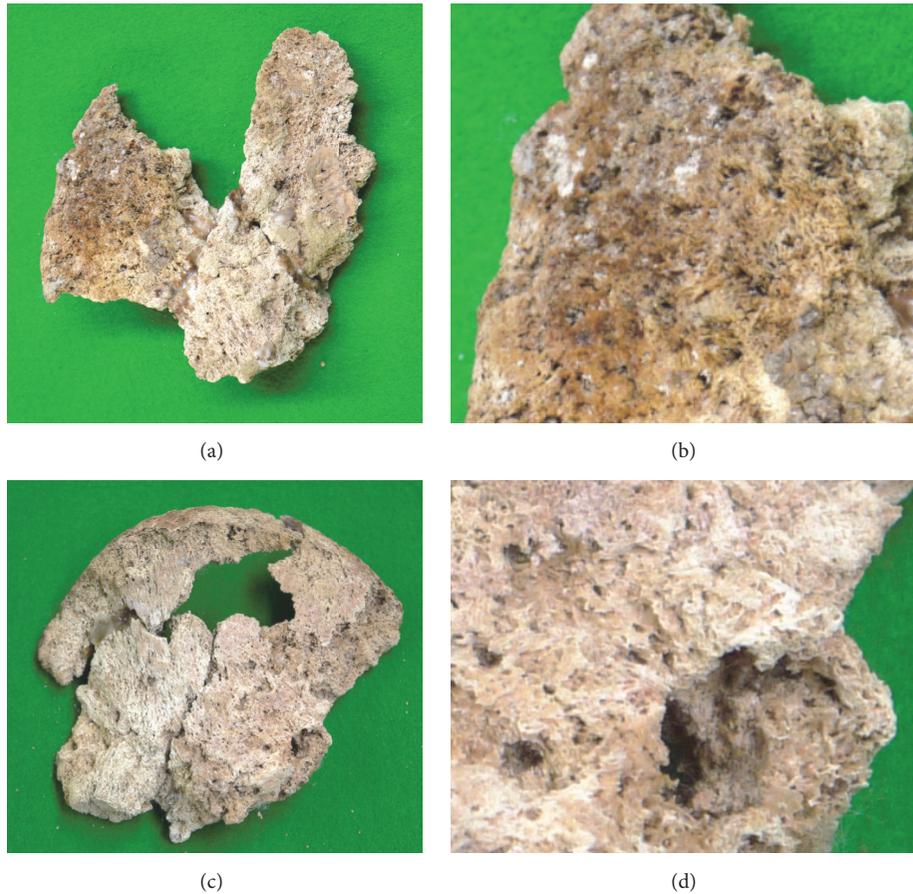


FIGURE 8: Photographs of the bilateral pelvic bones. The condition was poor on the right side (a, b) and left side (c, d). In the remaining region, either cortical or cancellous bone was replaced with osteoblastic lesions almost entirely from the surface to the inner region.

Acknowledgments

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Research Article

The Immunodetection of Non-Falciparum Malaria in Ancient Egyptian Bones (Giza Necropolis)

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The detection of falciparum malaria in ancient Egyptian remains had been performed by many authors using several methodologies including the use of rapid diagnostic tests. Through the immunochromatographic analysis of bony specimens from Giza skeletal collection dated to Old Kingdom, we provide first evidence of non-falciparum malaria in Ancient Egypt. The histidine-rich protein-2 (HRP2) specific to *Plasmodium falciparum* was absent in 100% of examined samples, while aldolase, common to the four types of plasmodial pathogens causing human malaria, was detected in 56% of individuals with no significant difference between the two tested social groups: high officials (HO) and workers (W). It is suggested that the main risk factor was the presence of residences near natural and artificial waterways, which allowed prolonged contact between the vector and human host.

1. Introduction

Human malaria is an infectious disease that is threatening human health in many parts of the world. Most cases are reported among children under 5 years. Many natural or human-made conditions can increase the chance of malaria transmission. The nutritional deficiencies problems may lead to the complication of the situation, especially among the most susceptible groups: children and pregnant women [1]. The most virulent form of human malaria is caused by *Plasmodium falciparum* that is responsible for the highest rate of mortality, while the diseases caused by *P. vivax*, *P. ovale*, and *P. malariae* are considered benign [2]. Abundance of *Anopheles* species responsible for malaria transmission in a given area is greatly influenced by climatic and environmental conditions. A great behavioral variety among different species is reported [3]. Social and cultural factors affect the biomedical burden of malaria; i.e., the perceptions of the disease in a given society, beliefs, cultural standards, and economic

and political circumstances influence the development of the disease, mortality, morbidity, and economic costs [4].

Few studies addressed the issue of malaria diagnosis in archeological human remains. They were carried on ancient Egyptian [5–13] and European remains [14–20]. With the exception of the studies carried by Gowland and Western [20] and Smith [12], all of these studies diagnosed the disease through the detection of biomolecules such as aDNA, antigens, and antibodies. Only five studies tried to diagnose non-falciparum forms of the disease: Biannuci et al., 2008; Pinello, 2008; Fornaciari et al., 2010; Gowland and Western, 2012; and Kendall et al., 2016.

The Giza Plateau contains not only the royal pyramids of the 4th Dynasty but also the tombs of high officials (HO) and workers (W) of the Old Kingdom [21] and Heit el-Ghurab or the workmen city of the 4th Dynasty that comprised an important Nile harbor [22].

The aim of this study is to estimate the prevalence of malaria among Giza community during the Old Kingdom

(4th-6th Dynasties) using malaria rapid diagnostic tests. Both HO and W were subjected to analysis.

2. Materials and Methods

2.1. Materials. The Giza bone collection is housed in a magazine in the Giza archeological site. The poor preservation state of many skeletons limited the number of examined individuals to 84 from a parent collection consisting of 305 skeletons. These bones were excavated from the following.

(1) The Western Cemetery: it is a cemetery of HO (4th-6th Dynasties) located west of the Great Pyramid. It was constructed to include the remains of the literate individuals who represented the elites such as princes, princesses, architects, scribes, priests, and temple officials [23]. It is argued that some owners of these tombs occupied important administrative positions in Heit el-Ghurab [24]. Skeletons under investigation were excavated by the mission of the Supreme Council of Antiquities (1989-1992) [25]. Only 54 individuals were available for examination.

(2) The Southeast Cemetery: it is the cemetery of W (4th-5th Dynasties) located southeast of the Sphinx. Buried individuals are those who worked not only in royal structures but also in the Giza HO tombs [21]. The Supreme Council of Antiquities started to excavate skeletons in 1990 [25]. Only 30 individuals were available for examination.

2.2. Methods. The eruption of the third molar was used to ensure that a skeleton is belonging to an adult according to Bass [26]. The poor preservation state of 12 skeletons hindered the estimation of age (Table 1).

84 bone specimens from the 84 selected individuals were used in the current study to extract malarial antigens. Mainly, thoracic and lumbar vertebrae were chosen; however, in case of unavailability of vertebrae other bony elements were used such as ribs, cuboids, patellae, calcanea, and tali.

Antigen extraction was performed in the Research and Conservation Centre, Ministry of Antiquities, according to the method described in the study of Fornaciari et al. [15] with the following exceptions:

- (i) The disinfection of the surface of bone elements before spongy bone extraction was performed using isopropanol swabs instead of UV radiation.
- (ii) A small sterile disposable medical blade was used to carefully remove a tiny area of the bone surface instead of using a drill. This aimed to ensure a better control and to avoid destruction of fragile archeological bones. A clean and disinfected obturator of an 11G bone marrow biopsy needle was used to obtain rough powder of spongy bone.
- (iii) Manual grinding of spongy bone powder was not performed under a laminar flow cabinet as the errors leading to false-positive or false-negative results can not be attributed to unsterile conditions [27].
- (iv) In each vial containing 50 mg of bony powder, 100 μ l of sterile physiologic solution (0.9% Na Cl) was

TABLE 1: Number of individuals in each social rank.

	HO	W	Total
Adults	45	27	72
Unknown age	9	3	12
Total	54	30	84

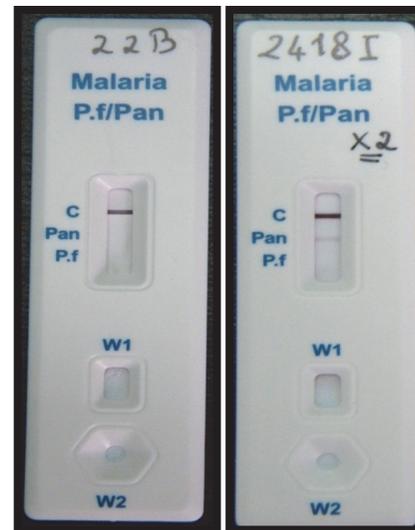


FIGURE 1: The test on the left is showing a negative result for malaria as only the control line (C) is appearing, while no colored lines are detected at the area of *P. falciparum* (Pf.) or that of Pan (the four types of malaria). The test on the right is showing a positive case of non-falciparum malaria as only aldolase band is detected.

added instead of 200 μ l to allow doubling the antigen concentration.

Malaria diagnostic test used in this study was ABON™ Plus Malaria *Pf/Pan* Rapid Test Device (Whole Blood) manufactured by ABON Biopharm (Hangzhou) Co. Ltd. This test can detect histidine-rich protein-2 (HRP2) specific to *P. falciparum* and aldolase common to all species. It was chosen according to the criteria cited in the report of the World Health Organization [28].

Using the IBM SPSS statistic software (version 22), statistical analysis was performed in the Centre for Static and Statistical Studies and Consultation, Institute of Statistical Studies and Research, Cairo University.

3. Results

The presence of malarial antigens was considered equivalent to the infection (Figure 1). Results indicated that 100% of tested individuals were negative for *P. falciparum* antigen, HRP2. The non-falciparum malarial species were detected in 56% of the tested samples, i.e., detection of aldolase in 47 individuals. Thus, no mixed infection with *P. falciparum* was reported (Figure 2).

The prevalence of non-falciparum malaria among HO and W was compared (Table 2 and Figure 3). Pearson's chi-squared test was used to evaluate the significance of difference

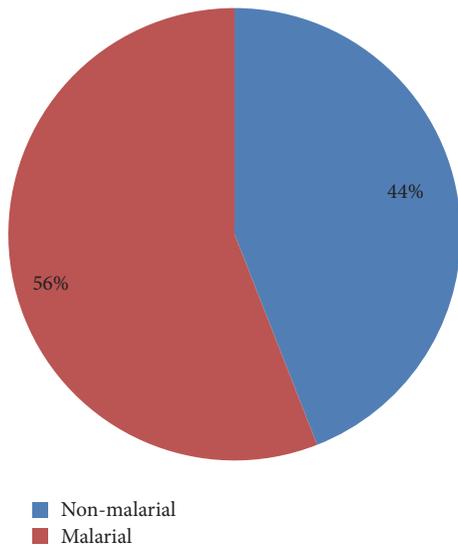


FIGURE 2: The prevalence of non-falciparum malaria among Giza population.

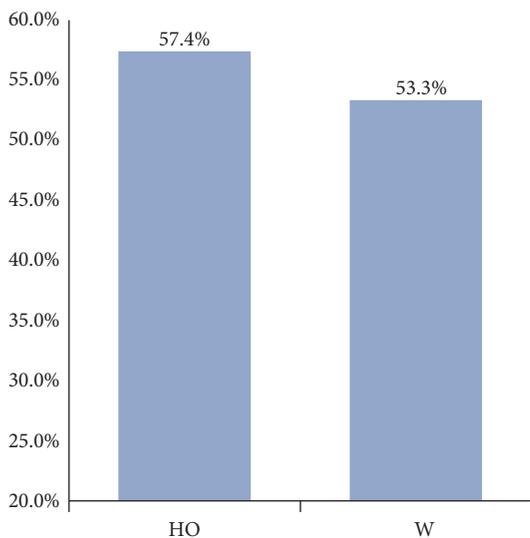


FIGURE 3: Comparison between prevalences of non-falciparum infection regarding social class.

TABLE 2: Malaria prevalence by social class.

Social class	Malarial	Non-malarial	Total
HO	31 (57.4%)	23 (42.6%)	54
W	16 (53.3%)	14 (46.7%)	30
Total	47 (56.0%)	37 (44.0%)	84

in malaria prevalence among social ranks. The difference was considered to be statistically significant below the cutoff value 0.05.

Although prevalence was higher in HO, no statistically significant difference between HO and W was reported ($p = 0.719$).

4. Discussion

The immunochromatographic technique was chosen to diagnose malaria in Giza population based on the positive results obtained from previous studies of Miller et al. [9], Cerutti et al. [6], Rabino Massa et al. [11], Bianucci et al. [5], and Fornaciari et al. [15] that detected malarial antigens in ancient human remains, especially that Cerutti et al. [6] and Fornaciari et al. [15] used bony samples; i.e., the stability of antigens over time and the ability to extract it from hard tissues had been proved. Also, although rapid diagnostic tests were invented to diagnose the disease in living patients, it was indicated that it can be used as an effective tool in paleopathological researches. In addition, the use of rapid diagnostic test in the current research was enhanced by its advantages reported by Moody [29]: it is a simple, rapid, economic, and sensitive technique. In contrast, many precautions must be considered in aDNA laboratories to avoid contamination [30]. Also, the aDNA techniques can not be considered economic [31]. In addition, the stability of aDNA is lower than proteinous molecules [32], while the detection of malarial hemozoin and antimalarial immunoglobulins in archeological bones are still virgin fields [33] (Kendall et al., 2010).

The results showed that 56% of the Giza population were infected with non-falciparum malaria, while no infection with falciparum malaria was recorded; i.e., only aldolase was detected. To our knowledge, this is the first time that a non-falciparum antigen is detected in archeological human remains, especially the ancient Egyptian bones. In addition, the previous efforts to detect the biomolecules of *P. vivax* in ancient remains did not meet expected success in spite of presence of historical evidences of malaria endemicity in studied populations, e.g., the researches of Pinello [18] and Kendall et al. (2012). Also, no paleopathological studies were performed to detect the presence of the aDNA of *P. ovale* or *P. malariae* in ancient bones until now. The findings indicating infection with non-falciparum malaria match the low mortality rate of subadults in the skeletal collection of Giza: the study of Smith [12] carried out on the commoners of Amarna, Egypt (18th Dynasty), where falciparum malaria was diagnosed indicated that the largest age group of Amarna bone collection was that of children as it reached 42%, while, according to El-Banna [25], the skeletons of subadults comprise only 11% of Giza bone collection.

Many precautions were considered to ensure the authenticity of results. To decrease the chance of having false-positive and false-negative results bones with sun bleaching, salt encrusting, mold growth, or consolidation materials were excluded from analysis. That is why only 84 individuals were chosen for analysis. In addition, a rapid diagnostic test with a high specificity was chosen. According to the kit enclosed instructions, the specificity of ABON Plus Malaria *Pf/Pan* Rapid Test Device is over 99% relative to microscopic technique of malaria diagnosis in clinical samples. The World Health Organization Product Testing Programme that is evaluating malaria tests manufactured by many companies around the world recommends using tests with false-positive rate less than 10%. The false-positive rate of ABON Plus Malaria *Pf/Pan* Rapid Test Device is 0.4% [28].

As explained by Sallers and Gomzi [19], there are no studies that indicated the mechanism of proteinous biomolecules degradation. Thus, negative results may represent “*an absence of evidence rather than an evidence of absence*” as cited by Bianucci et al. [5] and Bianucci et al. [14]. An argument against the diagenetic effect is the absence of HRP2 accompanied by the presence of aldolase with nearly the same rate in the bones of the two different groups buried in two different cemeteries and dated to the same historical period; i.e., findings reflected the spread of non-falciparum malaria in a specific geographical area during a specific era rather than a variation caused by diagenetic degradation of biomolecules.

Aldolase can persist for days after the clearance of the pathogen from the blood [34]. Thus, its presence in archeological bones can be interpreted as follows: the infected individual died while the pathogen was found in his blood or few days after the cure.

According to the immunochromatographic method used in the current study, it is not possible to determine which species of the three plasmodial pathogens causing non-falciparum malaria was responsible for the disease in Giza population, especially in the absence of textual sources that can be used as indicators for the disease in Giza. However, whatever the species responsible for malaria, the prevalence of the disease must account for the presence of an efficient *Anopheles* vector. As it was demonstrated by Pays [35] and Kenawy [36], there is no evidence indicating which species of mosquito were found in Ancient Egypt, although only two *Anopheles* species have been proved to be the vectors of malaria in Modern Egypt: *An. pharoensis* responsible for the transmission of *P. vivax* in Nile Delta and Valley and *An. sergentii* responsible for *P. falciparum* transmission in Oases [37]. The presence of non-falciparum malaria in ancient Giza may address a question about whether the vector in both ancient and recent eras is the same.

Since it is not possible to determine the species of *Anopheles* that was found in Giza, it is impossible to expect its behavior, e.g., whether the mosquito was exophagic or endophagic. However, it is expected that individuals experienced prolonged contact with the mosquito breeding sites, which allowed the transmission of the disease.

As it was explained by Strouhal [38], the abundance of mosquito breeding sites in Ancient Egypt can be attributed to the annual Nile floodings. This is supported by ancient texts such as in Sabbatani et al. [39]. Also, it is indicated by Noaman and El Quosy [40] that the artificial irrigation system that was practiced over the Dynastic Period involved the retention of the floodwater for about six weeks per year in agricultural lands of Delta and Nile Valley.

According to Love [41], Giza represented a part of the Egyptian capital that was located between the desert and the Nile. According to Lehner [24], a Nile channel was passing near Heit el-Ghurab. Also, this village comprised a major harbor and many waterways.

Since there is no significant difference between the prevalences among HO and W, it is argued that both were subjected to mosquito bites to the same extent due to their residence near the river, waterways and harbor. It is important to note that, according to Hawass [21], a group

of workers that occupied the barracks in Heit el-Ghurab worked through a system of rotation; i.e., farmers were brought from all Egyptian nomes every three months to participate in the project of pyramid building. Thus, the present study concludes that some workers buried in the Southeast Cemetery might be infected before coming to Giza due to their residence near the river bank and the application of basin irrigation system in their villages. This may raise the question about the prevalence of the non-falciparum malaria all over Egypt, not only in Giza.

According to Abu-Taleb [42], the social gap inequality increased gradually throughout the Old Kingdom due to the transition from an era of individualism to an era of feudalism. Food quantity and quality in Heit el-Ghurab indicated social inequality [43, 44]. The paleopathological studies of El-Banna [25] and Zaki et al. [45] carried out on the Giza skeletal collection suggested that male W were subjected to a higher degree of nutritional stress than male HO. The findings of the current research indicated that, despite diet stratigraphy, the disease prevalence among W was nearly equal to that among HO. However, this could partially be interpreted as a result of tetracycline ingestion. According to Gaillard et al. [46] tetracycline is used for malaria treatment since 1950. It was argued by Armelagos [47] that the detection of tetracycline in ancient human remains excavated from Dakhla Oasis, Egypt, which is attributed to beer consumption. Throughout the Dynastic Period, beer was available for all social classes and represented an essential part of all meals [48].

The study of Dahesh et al. [49] testing the prevalence of malaria among the recent inhabitants of a village in Fayoum, Egypt, showed that richer individuals showed a lower rate of infection than those belonging to lower rank because of enjoying better sanitation and hygiene and tendency to seek medical care at early stage of infection. The scarcity of evidences concerning cultural adaptation to malaria in Ancient Egypt does not allow an objective analysis of related socioeconomic factors, e.g., although it was suggested that the excavated bed of the Old Kingdom queen Hetepheres might be designed to bear a bednet [38] and the use of mosquito repellent was mentioned in a papyrus [35], there is no evidence about the use of bednets and repellents among HO and W.

Studies about fracture treatment and limb amputation that were carried out on the two social groups by Hussien et al. [50] and Zaki et al. [51] concluded that medical treatment was presented equally to both HO and W. Although this conclusion may contribute to the interpretation of findings of the present work, it is important to note that these studies did not deal with malaria; i.e., the perceptions of the disease and related cultural response cannot be estimated. The research carried out by O'Neill et al. [52] in Gambia manifested the importance of cultural beliefs about malaria: many individuals were not seeking professional medical care because some symptoms of severe malaria such as loss of consciousness were attributed to supernatural entities.

Generally, the absence of significant difference in malaria prevalence regarding social class in Giza reflects the ability of the disease to affect the whole society by the same degree; i.e., the socioeconomic factors did not affect the non-falciparum

malaria prevalence in ancient Giza, while it seems that the presence of residences near natural or artificial waterways represented a risk factor.

5. Conclusion

Using immunochromatographic analysis of archeological bony samples from Giza Necropolis, we provide first evidence of non-falciparum malaria prevalence in an ancient Egyptian population. This finding may enhance knowledge about the history of the disease that still affects many regions around the world.

Future work will involve studying the relation between the human-made environmental changes involved with urbanism and agricultural practices in Ancient Egypt and the disease prevalence. This will be performed through the comparison of results from Dynastic Period and Predynastic Period. This may include studying the socioeconomic burden of malaria among agricultural societies and hunter-gatherers.

It is recommended to perform aDNA studies on Giza population to detect the species and strains of the non-falciparum pathogen responsible for the disease. The chemical analysis of tetracycline in these archeological bones may extend our knowledge about the relation between dietary behavior and malaria. On a wider level, paleoclimatological researches may add new evidences helping to reconstruct the environmental conditions that permitted the reproduction and the spread of both the vector and the malarial pathogen.

Data Availability

The photos used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Lumbosacral Defects in a 16th–18th-Century Joseon Dynasty Skeletal Series from Korea

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Paleopathological evidence for congenital and degenerative disorders of the lumbosacral vertebrae is informative about ancient individual lifeways and physical conditions. However, very few studies have focused on the paleopathology of the lumbosacral vertebrae in ancient skeletal series from East Asia. One reason for the lack of studies is that skeletal samples from East Asia are typically insufficient in size to represent populations for comparative studies within the continent. Here, we present the first comprehensive analysis of lumbosacral defects in an East Asian human skeletal sample, examining occurrences of spina bifida occulta (SBO), lumbosacral transitional vertebrae (LSTV), and spondylolysis in remains from Joseon tombs dating to the 16–18th centuries in Korea. In this study, we present an alternative methodology for understanding activities of daily life among ancient Koreans through paleopathological analysis.

1. Introduction

Ancient human skeletal remains, when found in well-preserved condition, allow inferences about the etiology of pathology and make it possible to estimate health conditions in past communities [1]. Paleopathological evidence for congenital and degenerative disorders of the lumbosacral vertebrae is informative about ancient individual lifeways and physical conditions [2]. Pathological findings of the lumbosacral vertebrae, such as spina bifida occulta (SBO), lumbosacral transitional vertebrae (LSTV), and spondylolysis, have been observed in archaic hominins.

SBO is a congenital defect of the vertebral laminae associated with neural tube defects (NTD), or the failure of neural tube closure during embryonic development [3]. In SBO, the lamina have partial defects and the spinal cord is not involved. Both genetic and environmental factors such as folic acid deficiency during pregnancy affect the pathogenesis

of NTD [3]. SBO may be located at any level of the neural tube [4].

LSTV is a common developmental malformation in the area of lumbosacral transition [5, 6]. The prevalence of LSTV is reported to vary from 3.3% to 35.6% [7–9]. The most common type of LSTV is the sacralization of L5, which is caused by changes in the shape and size of the L5 processus costalis articulating with the upper edge of S1. The next most common transitional malformations are changes in the number of sacral vertebrae, which may be caused by the lumbarization of S1, by sacralization of Co1, or by the presence of a sixth sacral vertebra [5, 6]. Clinically, LSTV is associated with lower back pain (Bertolotti syndrome) [6, 10]. Degenerative changes in the intervertebral discs may cause lower back pain due to increased flexibility above the transitional vertebrae [6]. Additionally, LSTV significantly impacts the anatomy of surrounding structures in ways that are critical for spinal surgeons to recognize [11, 12].

Spondylolysis is a bony defect in the pars interarticularis of the human vertebrae [13]. This defect is associated with the unique lordotic posture of humans, an adaptation to upright walking [14, 15]. Spondylolytic defects are typically caused by congenital weaknesses or stress fractures [16]. Therefore, the majority of spondylolysis cases involve the lumbar spine, usually L5, and appear bilaterally. Males are affected more often than females because they often engage in more strenuous activities [14, 16, 17].

Lumbosacral defects have been reported in prehistoric and historic skeletal series throughout the world, except Asia [13, 18–22]. Existing reports of lumbosacral defects in Asia are limited to discussions of clinical epidemiology that focus on patient symptoms and complicated analyses of medical images [15, 23–27].

In this study, we present a comprehensive analysis of lumbosacral defects documented in human skeletal remains from Joseon tombs of the 16–18th centuries in Korea. Our data will facilitate a better understanding of the paleopathological prevalence of lumbosacral defects in past Asian peoples. We performed archaeological and anthropological analyses of the Joseon dynasty skeletal series and compare our results to those of previous reports in other countries during similar eras.

2. Materials and Methods

We examined a large series of human skeletons ($n = 198$) making up the Joseon Dynasty Human Sample Collection (JDHSC), which is maintained at Seoul National University College of Medicine, Korea. Most of the skeletons were collected from graves attributed to the Joseon dynasty. Korean archaeologists estimate that these Joseon graves date to the 16th–18th centuries. The Institutional Review Board (IRB) of Seoul National University Hospital confirmed that this study was exempt from board review (IRB no. 2017-001). We followed the guidelines of the Vermillion Accord on Human Remains, World Archaeological Congress [28].

Sex determinations were made on the basis of pelvic morphology, including examinations of the greater sciatic notch, preauricular sulcus, ischiopubic ramus, subpubic angle, subpubic concavity, and ventral arc [29, 30]. Ancillary indicators used for sex determination included characters of the skull, specifically the nuchal crest, the mastoid process, the supraorbital margin, glabella, and the mental eminence [31, 32].

Incomplete midline closure of the lumbosacral vertebrae indicated SBO, regardless of mesenchymal, osseous, or neural tissue origin during embryological development. Following previous definitions drawn from the literature, SBO at S3, S4, and S5 was considered within the limits of normal variation for the sacral hiatus [33]. LSTV was identified only if all of the lumbosacral vertebrae were present. After recording the number of lumbar vertebrae and sacral segments, the conversion of L5 or L6 into S1 was defined as sacralization and the reverse as lumbarization. The presence of an extra L6 with 5 sacral segments was defined as a form of LSTV in this study, including the lumbarization of S1 in cases of six sacral vertebrae and the sacralization of Col [5, 6]. Defects

TABLE 1: Frequencies of lumbosacral defects observed in this study ($n=198$).

	Number of findings (%)
Spina bifida occulta	15 (7.6)
Sacralization	10 (5.1)
Lumbarization	6 (3.0)
Spondylolysis	6 (3.0)



FIGURE 1: Sacral spina bifida (S1–S5) occulta in a male (Case no. 190).

of the pars interarticularis of the vertebrae were examined in cases of spondylolysis [15]. To prevent bias, we excluded cases of fresh spondylolysis that were difficult to differentiate from postmortem fractures of the pedicle.

We processed the data using descriptive statistics. Differences according to sex and age were evaluated using Fisher's exact test with a significance level of 5%, using R version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

The skeletal sample included 81 males, 68 females, and 49 indeterminates. Lumbosacral data are presented in Tables 1–4. The most frequent lumbosacral defect was SBO, followed by sacralization, and lumbarization or spondylolysis (Table 1). In cases of SBO, S1 was the most frequent defect site (8 of 198, 4.0%), followed by S2 (4 of 198, 2.0%) and L5 (2 of 198, 1.0%). Four male cases (1 case at S3, 3 cases at S4) and 3 female cases (2 cases at S3, 1 case at S4) were not regarded as SBO, but as congruent with normal variation of sacral hiatus. SBO was more frequently observed in males, especially at S1, than in females (Table 2). However, the difference between sexes was not significant by Fisher's exact test ($p = 0.143$). In one male case (no. 190), the SBO defect involved all segments of the sacrum as seen in Figure 1.

The prevalences of total LSTV were approximately 14% in males and 4% in females (Table 3). Males were more likely to exhibit sacralization of L5 and L6 than females ($p = 0.022$, data not shown) although the difference in cases of total LSTV (sacralization plus lumbarization) between sexes was not significant ($p = 0.088$). In the same skeletal series, we

TABLE 2: Descriptive statistics of spina bifida occulta (SBO) in Joseon males and females.

	Number of SBO occurrences/total (%)			P value*
	Males	Females	Indeterminate	
Spina bifida at L5	1/81 (1.2%)	1/68 (1.5%)	0/49 (0.0%)	1.000
Spina bifida at S1	6/81 (7.4%)	1/68 (1.5%)	1/49 (2.0%)	0.126
Spina bifida at S2	2/81 (2.5%)	1/68 (1.5%)	1/49 (2.0%)	1.000
Spina bifida at S1–S5	1/81 (1.2%)	0/68 (0.0%)	0/49 (0.0%)	1.000
Total	10/81 (12.3%)	3/68 (4.4%)	2/49 (4.1%)	0.143

* probability value of Fisher’s exact test between the sexes.

TABLE 3: Descriptive statistics of lumbosacral transitional vertebrae (LSTV) in Joseon males and females.

	Number of LSTV occurrences/Total (%)			P value*
	Males	Females	Indeterminate	
Sacralization at L5	7/81 (8.6%)	1/68 (1.5%)	0/49 (0.0%)	0.071
Sacralization at L6	2/81 (2.5%)	0/68 (0.0%)	0/49 (0.0%)	0.501
Lumbarization at S1	1/81 (1.2%)	1/68 (1.5%)	2/49 (4.1%)	1.000
Extra L6 with 5 sacral segments	1/81 (1.2%)	1/68 (1.5%)	0/49 (0.0%)	1.000
Total	11/81 (13.6%)	3/68 (4.4%)	2/49 (4.1%)	0.088

* probability value of Fisher’s exact test between the sexes.



FIGURE 2: Extra (sixth) lumbar vertebra (left) with normal sacrum consisting of 5 sacral segments (right) in a female (Case no. 113).



FIGURE 3: Spondylolysis of L5 with six lumbar vertebrae in a specimen of unknown sex and age (Case no. 289).

also note that two cases, one female (no. 113) and one male (no. 162), exhibited extra L6 alongside 5 sacral segments (Table 3, Figure 2). These cases were considered to represent the lumbarization of S1 among the six sacral vertebrae that were present.

The prevalence of spondylolysis according to sex is shown in Table 4. Overall, the most frequent defect site was L5 (4 of total 198, 2.0%) but no defects were found in the sacrum. There were no significant differences between sexes (Table 4). Among the sex-indeterminate skeletons, we identified a unique case (no. 289) with an extra L6 and spondylolysis at L5 (Figure 3).

4. Discussion

NTD, including SBO, are some of the most common types of congenital malformations, occurring at 21 to 28 days

after conception [34]. The general prevalence of NTD is estimated to be 0.51–4.2 per 1000 live births, stillbirths, and pregnancy terminations in the modern period [35, 36]. The birth prevalence of spina bifida may differ considerably depending on geographical and population origin [37].

SBO is the most common developmental defect of the vertebral column observed in historical skeletal series, occurring frequently at the lumbosacral border, with reported incidences of 5.2% to 26.0% [13]. In our study of Joseon skeletons, we also found that SBO was the most commonly observed pathology in the lumbosacral vertebrae. There are significant temporal and geographic influences on maternal nutrition, including folic acid supplementation, affecting the etiology of spina bifida [34]. When we compared data for the

TABLE 4: Descriptive statistics of spondylolysis in Joseon males and females.

	Number of spondylolysis occurrences / total (%)			P value*
	Males	Females	Indeterminate	
Spondylolysis at L2	1/81 (1.2%)	0/68 (0.0%)	0/49 (0.0%)	1.000
Spondylolysis at L4	1/81 (1.2%)	0/68 (0.0%)	0/49 (0.0%)	1.000
Spondylolysis at L5	1/81 (1.2%)	2/68 (2.9%)	1/49 (2.0%)	0.592
Total	3/81 (3.7%)	2/68 (2.9%)	1/49 (2.0%)	1.000

*Probability value of Fisher's exact test between the sexes.

Joseon skeletal series to the results of previous studies examining different countries and different historical periods, SBO tended to have higher prevalence in the Joseon sample than in most historical populations, excepting medieval Slovakia (Table 5). The prevalence of SBO in Slovakian skeletal series [13] may have been higher than the prevalence of SBO in Korea during the Joseon dynasty, because the majority of lower sacral segment defects (S4-S5, S3-S5) were regarded as SBO, which was in turn defined as being with the range of normal variation for the sacral hiatus. Taken together, these observations indicate that the prevalence of SBO among the Joseon people is one of the highest ever observed among ancient skeletal series. Neurological defects, particularly spina bifida, have actually revealed a significant decrease over time due to the supplementation of food with fortified folic acid worldwide [38]. Therefore, the high prevalence of SBO in our Joseon dynasty sample might have been caused by maternal folate insufficiencies that were more serious than in other populations.

The lumbosacral region is the most frequent site of border shifting [13]. Abnormal cranial shifting leads to the sacralization of the fifth lumbar vertebra, while caudal shifting leads to lumbarization of the first sacral vertebra. In both cases, the defect may be complete or incomplete, unilateral or bilateral, and symmetrical or asymmetrical. According to previous studies, the general prevalence of LSTV ranges from 4% to 35.6% in different populations, and males are more frequently affected than females [7-9]. Compared with samples from different countries but of similar era, differences of prevalence of LSTV between Joseon-Korea and other medieval countries prove to be minor, with a slight tendency for the prevalence of LSTV to be higher in males than in females, a pattern that holds true throughout a variety of countries (Table 5).

In the literature, spondylolysis is described as ossification union failure or the fracture of the pars interarticularis of the vertebra, resulting in separation of the vertebra into two parts. This separation occurs most commonly in S1 and L3-L5 [13]. The prevalence of lumbar spondylolysis is estimated to be 5% in the general population but can be as high as 63% in people who participate in certain sporting activities and also varies between populations [14, 16, 39]. For example, 13% of a sample of Canadian Inuits exhibited spondylolysis, while the prevalence was 18.5% in a sample of Archaic Indians [13]. Approximately 20.7% of Japanese athletes are affected by spondylolysis [40]. Lumbar spondylolysis is thought to result from stress fractures of the pars interarticularis that

occur frequently in athletes. The Joseon sample examined in this study was assumed to represent individuals only infrequently engaged in mechanically demanding activities, with a total incidence of spondylolysis (3.0%) lower than that of any other known ancient skeletal series (Table 5). The prevalence is also low compared to modern Koreans, in which population values range from 5.9% to 9.1% [14, 41]. The low frequency of spondylolysis observed in the Joseon sample suggests a lack of heavy physical labor in this population [29]. As the individuals in the sample primarily represent the upper and ruling classes of the Joseon kingdom [42], the relatively low prevalence of spondylolysis we observed could reflect socioeconomic differences. However, comparisons of disease in different skeletal series must be approached carefully if research methods used have not been standardized [34].

5. Conclusion

Very few studies have focused on the paleopathology of lumbosacral vertebrae in ancient skeletal series from East Asia. One reason for the lack of studies is that skeletal samples from East Asia are typically insufficient in size to represent populations for comparative studies within the continent. This paleopathological study of lumbosacral pathologies in a large sample from the Joseon era is thus significant. Our results suggest that chronic scarcities of folic acid were prevalent during the Joseon dynasty, as evidenced by the high incidence of SBO, similar to other medieval samples. In contrast, the relatively low prevalence of spondylolysis may reflect a lack of strenuous physical labor among the ruling classes of Joseon society. Differences in the prevalence of LSTV between Joseon people in Korea and medieval samples from other countries are minor, indicating that genetic causes are unlikely. This study contributes to our understanding of daily life among ancient Koreans through paleopathology.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

TABLE 5: Prevalence of lumbosacral malformations in different samples during similar eras.

Country	Century	Spina bifida occulta			Sacralization			Lumbarization			Spondylololysis		
		Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
Joseon Korea (This study)	16-18th	12.3	4.4	7.6	9.9	1.4	4.5	4.9	2.9	4.0	3.7	2.9	3.0
British [18]	10-19th	-	-	-	-	-	-	-	-	7.0	14.5	8.9	11.9
Bulgaria [19]	9-15th	6.6	0	3.8	-	-	2.9	-	-	-	-	-	-
France [21]	5-11th	-	-	-	-	-	3.3	-	-	3.3	-	-	10
Hungary [22]	10-17th	7.1	3.3	4.2	2.7	0.9	1.4	3.5	-	1.4	-	-	-
Romania [20]	15-19th	5.2	1.9	3.8	-	-	-	-	-	-	-	-	-
Slovakia [13]	11-12th	26.0	19.0	22.0	15.0	2.0	8.0	2.0	2.0	2.0	8.0	8.0	7.0

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Review Article

Paleopathological Considerations on Malaria Infection in Korea before the 20th Century

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Malaria, one of the deadliest diseases in human history, still infects many people worldwide. Among the species of the genus *Plasmodium*, *P. vivax* is commonly found in temperate-zone countries including South Korea. In this article, we first review the history of malarial infection in Korea by means of studies on Joseon documents and the related scientific data on the evolutionary history of *P. vivax* in Asia. According to the historical records, malarial infection was not unusual in pre-20th-century Korean society. We also found that certain behaviors of the Joseon people might have affected the host-vector-pathogen relationship, which could explain why malarial infection prevalence was so high in Korea at that time. In our review of genetic studies on *P. vivax*, we identified substantial geographic differentiation among continents and even between neighboring countries. Based on these, we were able to formulate a strategy for future analysis of ancient *Plasmodium* strains in Korea.

1. Introduction

Globally, malaria is the fifth deadliest disease, infecting approximately 200 million people worldwide [1–3]. Malarial infection is mediated by the arthropod vector *Anopheles* mosquito. The *Plasmodium* parasite has a complex life-cycle of sexual reproduction inside the mosquito vector and asexual stage in the vertebrate host. In brief, malarial sporozoites are inoculated into human hosts when a mosquito bites them [4]. After a dormant phase, they differentiate into merozoites for release into the bloodstream, upon which they invade erythrocytes (the beginning of asexual multiplication). The bursting of infected red blood cells (RBCs) by merozoite multiplication is responsible for the typical malarial fever [4]. Some of the merozoites then develop into gametocytes, which are taken up by a female mosquito [4]. Sexual reproduction in the anopheline mosquito is followed by sporozoite migration into the salivary gland, from which they are inoculated into a vertebrate host, thus beginning a new cycle of malarial infection [4].

In general, five species of genus *Plasmodium* are known to cause malaria: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* [5]. Recent malaria outbreak in Brazil has also been traced to new zoonotic transmission of *P. simium* from monkey [6]. Among them, *P. vivax* and *P. falciparum* are the most commonly detected causative pathogens of human malaria. Clinical manifestations of uncomplicated malaria are nonspecific: headache, fever, malaise, myalgia, nausea, vomiting, and abdominal pain. Rare cases of malaria show severe manifestations including anemia, thrombocytopenia, pulmonary edema, renal failure, hepatic dysfunction, and splenic rupture [7].

Although malarial species share typical signs and symptoms such as undulating intermittent fever, they also have different traits depending on each subtype. *P. falciparum*'s symptoms are serious enough to show the highest mortality rate whereas *P. vivax*, *P. malariae*, and *P. ovale* exhibit generally nonfatal clinical courses [5]. The geographical distribution of each *Plasmodia* subtype differs as well. *P. falciparum* is more

prevalent in tropical or subtropical zones including Sub-Saharan areas but relatively absent in temperate countries [8]. Meanwhile, *P. vivax* generally infects human populations in temperate and tropical zones but is not so prevalent in Sub-Saharan Africa [1, 3, 9]. *P. vivax* malaria was endemic even in some high latitude countries (Finland and Russia, etc.) at certain points in history [4, 10, 11].

As malaria historically has been, and continues to be, one of the most serious diseases, it has attracted the attention of many paleopathologists. Studies on ancient malarial infection have been conducted by various methods such as osteoarchaeological and biomedical approaches [12]. As chronic-stage *vivax* malaria was generally known to induce anemia, further causing porotic hyperostosis (PO) or cribra orbitalia (CO) in the cranium [10], anthropologists have searched for the presence of PO or CO in skeletal remains as indirect evidences of malarial infection [5, 12–15]. Nevertheless, PO or CO has clear limitations with respect to its application to the study of ancient malaria because *Plasmodium* infection is not the only cause of them [12, 16]. Other pathologies such as inherited hemolytic anemia, scurvy, or malignancies are also known to induce the same skeletal changes of PO or CO [5, 12].

In recent years, the paleopathological study of malaria has been revolutionized by successful applications of immunological and ancient DNA (aDNA) analyses to archaeological specimens. To detect malaria-related proteins, researchers performed the dipstick assay or new-generation immunoassays on ancient mummies [17–19] or skeletons [20, 21]. The immunological assay became an effective screening method to secure the evidence of ancient malarial infection [5]. Also, *Plasmodium* aDNAs reportedly have been obtained from Egyptian mummies [2, 22–24], an infant skeleton dating to ancient Rome [25], 15th-to-19th-century infant of Bavaria [26], and 1st-to-2nd-century adult skeletons of Italy [27]. As a paleopathological tool, aDNA analysis is useful for confirming the presence of malarial genomes remnant in archaeological specimens as well as for revealing the origin and dispersal of the protozoan parasite in evolutionary history [28].

Although immunological and molecular analyses have become more reliable tools for the study of ancient malaria, the data obtained to date are not sufficient in terms of quantity and quality [16]. Moreover, since previous studies have focused mainly on ancient Egyptian, Roman, and Renaissance European remains thus far [5], such information as has been obtained from malaria aDNA reflects a serious geographical bias. Extensive geographic sampling is thus necessary in order to understand the demographic history of malaria much more comprehensively and clearly [4, 29–31]. Like the other continents, Asia is also a region where malaria has historically been epidemic and endemic. In several Asian countries, many people continue to have suffered from and even died of *Plasmodium* infection. Nonetheless, most of the requisite paleopathology still remains to be revealed in Asia as few medical studies on the ancient malaria have been reported in the area. Herein, then, we offer this historical review as a fundamental basis for future research of ancient malaria infection in Korea and other Asian countries.

2. Origin and Dispersal of Vivax Malaria Parasite

Parasitologists have speculated that human malaria might have been transmitted from nonhuman primates by a host-switch event [11]. Initially, they presumed that *P. falciparum* was transmitted from chimpanzees and gorillas in Africa [18] while *P. vivax* originated from another nonhuman primate, possibly macaques, in Southeast Asia [4, 32, 33]. However, this hypothesis is seriously challenged nowadays by the genetic analysis of malaria worldwide. Alternatively, a recent study revealed that both *P. falciparum* and *P. vivax* originated in Africa and that *P. vivax* transmission to human beings might have occurred much earlier than *P. falciparum* did by a host switch [34].

According to the estimated time to most recent common ancestor (TMRCA), the ancestor of the extant *P. vivax* populations existed between 50 and 550 ka before the present [3]. In a demographic history inferred from the *P. vivax* genome analysis, the global population size of vivax malaria might have expanded slowly until about 60 ka BP, which is closely consistent with the demographic history of mankind [3, 35]. Once the divergence of African and Eurasian *P. vivax* populations occurred at about 51 ka BP, the latter appears to have undergone a rapid exponential increase in population size [3, 35]. Among Eurasian vivax malarias, the East Asian variety might have experienced a distinct pattern of population growth [3]. The population of East Asian variety might have been relatively stable in its expansion until approximately 10,000 years BP [36, 37]. It then began to increase rapidly once rice and millet started to be domesticated in the area and sustained such increase, without tapering off, until the present [3]. The inferred hypothesis is suggestive of the detailed evolutionary history of vivax malaria in East Asia [3].

In the phylogenetic tree of *P. vivax* worldwide, two divergent groups were identified: a large star-like cluster and a divergent cluster [3]. The latter was composed of two subclades with different geographical distributions: “Asia a” of Central China and “Asia b” of China, Korea, and Indonesia. The divergent East Asian *P. vivax* lineage was connected to the large star-like cluster by a group of haplotypes found in Southeast Asia [3]. Based on the phylogenetic analysis, East Asian *P. vivax* might have been split from all other vivax malaria and developed a distinct demographic history for at least 121 ka [3]. Meanwhile, mutations of *P. vivax*-resistant RBCs (Duffy-negative phenotype) occurred in Sub-Saharan peoples [38]. Due to the mutations, vivax malaria disappeared completely from the area until the reintroduction of *P. vivax* to East Africa by sea-going traders from Asia [39].

3. History of Malaria Infection in Korea

The historical record is important for understanding the pattern of malarial infection in ancient civilizations [27]. In the classical period of Greece, Hippocrates famously described the typical undulating fever, a very suggestive sign of malarial infection [5, 39]. Historians believed that malaria became hyperendemic in Europe by its spread around the Mediterranean area, next along the riverbanks of the Rhine,

Danube, and Rhone and then further to Northern Europe, while accommodating to colder climatic conditions in those areas [40]. Historical studies have shown that malaria became remarkably prevalent in the marshy areas of Northern Europe in the Early Middle Ages [5, 40, 41]. By the Later Middle to Early Modern Ages, except for Iceland, plenty of reports on malaria were available from every corner of Europe (including the North Sea, Germany, Anglo-Saxon England, and even Scandinavian countries) [40, 42, 43]. In a sense, malaria appears to have been a much more serious disease than even the Plague [40, 44].

Malaria must have been endemic in East Asia from ancient times as well, as descriptions about malaria-like symptoms can be seen in Chinese historical records [45]. Although Korea had been in close interaction with China from earliest times, in Korean history, the first recorded case of malaria occurred only in the Goryeo Dynasty (918-1392 CE) [46]. In a 14th-century record, a Joseon King's mother (Joseon Dynasty: 1392-1910 CE) was seriously infected with malaria and eventually died of it [47]. Over the following centuries, a wealth of records on the typical signs and symptoms of malarial infection (intermittent fever, repeated every third day) can be found in the Korean historical literature [47]. As most malarial infection in modern Korea has been revealed to have been caused by *P. vivax* [48], the Joseon people might have suffered from the same *Plasmodium* subtype. Notwithstanding the benign traits of *P. vivax*, relapsed infection typically might have exhausted people, often eventually killing them, as seen in similar clinical reports today [3, 49-53].

Before the first modern medical record on malaria in Korea (1886), prevalences of malarial infection could not be reliably calculated. In the *First Annual Report of the Korean Government Hospital, Seoul*, Dr. Horace Newton Allen described "endemic intermittent fever" (possibly malaria) as the most commonly observed sign among Korean patients who visited his hospital [54, 55]. According to him, in the late 19th century, hyperendemic malaria posed a serious threat to Koreans throughout the entire Joseon Kingdom. How, exactly, did malaria show such a high infection prevalence in Joseon society? In general, wetlands such as scattered swamps, bogs, and river valleys have been important habitats for anopheline mosquito breeding. As wetlands were distributed widely in Korea at that time, they must have been integral to the high malarial transmission rates [34, 40, 56-63].

In malariology, however, the waxing and waning of malarial infection in a specific area cannot be explained so simply. In addition to wetlands, environmental alteration or degradation due to human activity also has a great influence on the density and activity of mosquito populations and, further, on the prevalence of malaria itself [64-66]. Table 1 summarizes the anthropogenic causes of malaria currently recognized by scholars. As is apparent, people's efforts to exploit environments often induce outbreaks of malaria [40, 60, 64, 67]. Indeed, agricultural development and malaria are highly correlated in human history [60, 68-72]. The expansion of irrigation facilities, the reclamation of wetlands, economic specialization in agriculture, the simplification of crop types, enlargements of rice paddies, high population

densities, deforestation, and still other malaria-inducing factors have been commonly cited (Table 1).

We do not yet know whether the close relationship between environmental change by agriculture and malarial infection is a universal phenomenon beyond certain temporal and spatial limits. In a recent cross-national analysis, however, correlations among anthropogenic activity, mosquito population sizes, and malaria rates were seen to have been common in many parts of the world [64]. The findings of Table 1 can thus be applied to our conjecture about Joseon society's vulnerability to malarial infection. In our careful examination of the Joseon records, we found many similar malarial-infection-facilitating situations to those noted in Table 1. The situations in Joseon society are summarized in Table 2.

In brief, the 15th to 19th centuries in Korean history were a turbulent and dynamic period during which the Joseon people were highly motivated to be involved in agricultural innovation, thereby eventually effecting major changes in their sociocultural environment (Table 2). By infusion of labor and capital investments into land development, the state of the agricultural techniques was advanced. By clearing every corner of wasteland and reclaiming wetlands, huge areas of farmland in the Kingdom were newly opened up [73-77]. Farmers cleared slash-and-burn fields even up to the tops of mountains [63, 73, 76]. By the end of the Joseon Dynasty, there was virtually no land remaining that had not been utilized for farming purposes (Table 2).

On such lands, Joseon farmers planted crops. Rice was very popular, becoming the most preferred crop by the late Joseon Dynasty [73]. To meet growing market demand for rice, farmers hastily turned their existing dry fields into rice paddies [54, 63, 73-75, 78, 79]. To supply enough water for rice cultivation, irrigation systems comprised of reservoirs and dammed pools were newly constructed in the Kingdom [54, 63, 74, 75, 78, 80]. Due to such increased agricultural productivity during the 15th to 19th centuries, the population of the Joseon Kingdom soared [73, 76]. All of these changes meant that the Joseon people came to live more and more in highly populated villages, towns, and cities around which rice paddies, reservoirs, and dams were scattered (Table 2). Certainly, as long as this new situation continued, malarial prevalence was by no means lowered. In a sense, intensive farming appears to have been a necessary evil for the Joseon people, as, notwithstanding the malaria-inductive environments thus created, the increased food production potentiated and achieved thereby was a great economic as well as social boon to the Kingdom.

From the late 19th century, the diagnosis and treatment of malaria began to be performed by specialists in Western medicine. In 1913, for example, an intermittent fever observed among Korean patients was finally confirmed by a modern microbiology technique to be *Plasmodium* infection [54, 81]. During the Japanese colonial period, however, significant reduction of malarial incidence proved difficult, as the environmental conditions associated with agriculture remained the same. Since the end of World War II and subsequent US army administration, malarial infection as well as its

TABLE 1: The factors proven to relate with high prevalence of malaria infection.

Factors	Details	References
Marsh, wetland, coastal area, swamp, etc. as the possible source of malaria infection	In the African countries, anopheline larvae were abundantly found in the swamps, and topographic wetness (of wetlands) was strongly associated with the spatial distribution of malaria infection cases. The control of wetlands is important for malaria elimination. In the late Bronze to early Iron Age Europe, the occupation of the coastal marshes paved the way for the spreading of malaria. Mosquito larvae were able to grow up in stagnant pools and ditches of North Sea or Anglo-Saxon England coast marshes. In European history, the coastal marshes were generally hyperendemic for malaria during 16th to 18th century.	[34, 40, 56–62]
Expansion of irrigation facilities	In Ancient Egypt and Rome, Fayum area became the granary by repeated projects of large-scale land reclamation and construction of canal system. <i>Anopheles</i> vector breeding was directly or indirectly linked to the presence of extensive irrigation system. Fayum was seriously exposed to the hazards of malaria by increasing contact between human beings and mosquitoes that were bred in the newly developed irrigation system. The linkage of land reclamation and malaria infection was proven in East Africa. For instance, the elimination of papyrus from the wetlands during land reclamation promotes the breeding of mosquitoes and malaria infection. Drainage ditches in newly claimed agricultural land were also the most common breeding site for mosquitoes. In brief, the land reclamation was to foster mosquito reproduction by reduced vegetation cover and the elevation of the temperature at breeding site.	[2, 60]
Reclamation of wetland for agriculture	Epidemiological study showed that malaria transmission and intensified crops are highly related. The incidence of malaria is about ten times higher in cereals-cultivation area than in areas with less cereals. Intensity of crop cultivation is highly associated with exacerbated human risk of malaria. Specialization in agriculture initially influences on forest loss, and further induces malaria infection (by reducing the biodiversity due to a replacement of huge variety of vegetation with nonnative crops).	[2, 64, 124–127]
Intensified crop cultivation or economic specialization in agriculture	The rice paddies provide abundant breeding opportunities for malaria mosquitoes. It also becomes a challenging site for vector control. Improper drainage from rice paddies caused Anopheline mosquitoes to breed. Mosquito control agents are difficult to be applied to rice paddies. Rice cultivation has a deep-rooted relationship with malaria transmission.	[124, 128–133]
Rice cultivation		

TABLE 1: Continued.

Factors	Details	References
Deforestation	<p>The pupation rate of <i>Anopheles</i> mosquito was the highest in the samples collected from deforested areas. Land cover pattern is a key factor that influences the habitat for malaria mosquitoes. Relationship between deforestation caused by a small-scale farming and <i>Anopheles</i> mosquito breeding was evidently proven in Amazon region.</p> <p>In a structural equation model across 67 (developing) nations, positive association was observed between deforestation rates and malaria prevalence. In Sub-Saharan countries, living in the land without trees led to the increased risk of malaria infection. The relationship between land cover and the reproduction of malaria vector mosquitoes was also shown in Western Kenyan Highlands.</p> <p>Epidemiological aspects of ecosystem change (deforestation) and mosquito habitat proliferation (increased levels of larvae, mosquito populations, and actual malaria rates) have been studied extensively. Significant relationship was observed between the percentage of forest cover loss and higher infection prevalence of malaria.</p> <p>Deforestation impacts malaria prevalence by multiple mechanisms: increase in the sunlight amount, warming temperature ideal for the pupation of malaria vector larvae, standing water after clearing terrain, the land becoming flatter and more likely to store water, which is typically less acidic and more conducive to <i>Anopheles</i> larvae development.</p> <p>When the forest is replaced by new croplands, the plants still provide the bushy cover for mosquito proliferation, making the malaria infection prevalence higher.</p>	[60, 64, 65, 134–144]
High population density	<p>In developing countries, rural population growth and needs to increase food production induce the forest loss, further influencing malaria infection. Using the timbers for building and fuel wood is also one of the key causes of deforestation. Growing rural population also tend to live closer to the natural habitats of mosquitoes, further experiencing risk of malaria infection.</p> <p>In medieval city of Groningen, 10 percent of the urban population died while the surrounding countryside showed a death rate of 5 percent.</p>	[40, 64, 145–147]

TABLE 2: Historical findings possibly related to the malaria outbreaks in Joseon society.

Changes in history	Date	Historical details	References
	15th to 18th century	Piling up reservoirs or dams to turn wetlands into rice paddy fields. At the late 17th to 18th century, rice cultivation became dominant in the agriculture of Joseon Dynasty.	[63, 73]
Increase in the ratio of rice paddies to total cultivated land	18th to 19th century	Changing cultivated dry fields to the rice paddies (42.9% in 1759 to 68.6% in 1901). One-third of rice paddies of Joseon Kingdom was created at this period.	[73–75, 78, 79]
	Japanese colonial period	Changing the dry field to rice paddies still continued in colonial Korea. <i>Anopheles sinensis</i> , the vector mosquito of malaria in Korea, usually propagates in the stagnant water of rice paddies. Malaria infection was thus very common in the places.	[54, 79]
	US military administration	Trying to get rid of such mosquito propagation spots by making gutters to drain the water.	[79]
Construction of reservoirs or dams	15th to 19th century	From the late 15th century on, the irrigation system such as reservoirs and dams were actively built in Korea. The water in the dams could be drained into the paddies for rice cultivation. The construction and maintenance of irrigation system was governed by government or gentries in countryside (16th century). In the early 19th century, the number of reservoirs or dams reached as many as 5,960 in Korea. The construction of dams and reservoirs reached the peak in the early 19th century, maintained even in Japanese colonial period.	[54, 63, 74, 75, 78]
Simplification of crop types	Since 18th century	To benefits from higher productivity of rice farming and respond the market demands much efficiently, the proportion of rice in cereal cultivation has been greatly expanded.	[73]
Clearing and reclamation of the land; Slash and burn in mountain area	15th to 18th century	Large-scale reclamation was taking place in abandoned land, wetland, coastal, and low-lying areas at river or stream basins (15th to 17th century). Slash and burn increased in mountainous area (since 17th century). Previous ranch and isolated islands were also turned into farmland. The nonfarming backdrops almost disappeared throughout the country after mid-17th century.	[73–77]
Deforestation	Since 17th century	Population growth in Joseon society led to increased demand for forest products. Serious deforestation at this stage was also caused by slash-and-burn farming (after 17th century on). At this stage, approximately 40–50 percent of the cropland in Joseon Kingdom was prepared by slash-and-burn farming, causing deforestation in the mountain area. In mid-17th century, no matter how deep the mountain area was, there was no place where no cropland was. Deforestation induced frequent landslides and floods.	[63, 73, 76]
	Colonial period	Deforestation appears to have induced malaria in colonial period Korea.	[54]
	Mid-16th century	9 to 10 million people in Korean peninsula.	[73]
Population increasing	Late 18th century	Population in Korean peninsula reached 15 million. The increase in population at this stage appears to have been due to the high productivity of rice cultivation in Korean peninsula at that time.	[73, 76]

management underwent a revolutionary change in Korea. The US army, which had experienced many deaths from malaria in the course of the war with Japan, was able to establish an effective means of controlling the *Plasmodium* infection in Asia [79]. Whereas the Japanese during the period of their colonial rule of Korea preferred a strategy entailing curing of malaria patients by quinine treatment at the onset of the disease, the US military administration adopted a far more aggressive and highly effective policy of controlling malaria through the use of insecticides [79].

However, we must also consider the possibility that malarial prevalence in the country was not reduced by antimalarial medications or insecticides alone. This idea is supported by instances in European history. In the 18th to 19th centuries, malaria was still prevalent in Europe, though it rapidly declined thereafter, finally disappearing from most regions by the 1930s [82]. The retreat of malaria from Europe was not the result of medical or chemical innovations, as no such deliberate countermeasures (entailing use of quinine or insecticide, etc.) had been pursued at that time [83]. Rather, another factor has been proposed to explain the decline of malaria in Europe: socioeconomic progress [84]. Many studies in fact have shown a positive correlation between malaria and poverty [85, 86]. Furthermore, it has been established that malaria was active for much longer periods of time in regions where modernization was delayed [40]. In Finland, long-term social changes such as land consolidation, decreasing household size, fewer interactions between families, and the transition from extended family to nuclear family have been posited as causes of malaria's decline during the past 200 years [83]. In fact, the combined effects of social innovations and improved standards of living were decisive in controlling and eventually eradicating malaria in Europe [40, 60]. Arguably, this explanation is applicable to the decline of malaria in South Korea as well. In that country, malaria was eradicated in the 1970s by the cooperative efforts of the World Health Organization (WHO) and Korea's National Malaria Eradication Program [48, 87]. Significantly, this corresponded to the period of rapid industrialization by which the living standards of the Korean people were remarkably improved. In short, socioeconomic development in South Korea might have made a great contribution to the eradication of malaria in that country.

Unfortunately, the reemergence of malaria after long-term eradication is not a rare phenomenon in the world [3, 88]. In 1993, after more than two decades of malaria-free status, a new Korean malaria patient was reported among soldiers who had served near the Demilitarized Zone (DMZ) in South Korea [48, 89]. At the time, as North Korea was suffering malarial infection, the new patient was thought to have been infected by an *Anopheles* mosquito migrating from the north [87, 90]. In fact, this cannot explain everything about the reemergence of malaria in South Korea, as, nowadays at least, foreign travelers and workers from malaria-endemic regions are commonplace [87]. Today, malaria is once again an endemic disease and a source of public concern in South Korea, as cases of malarial infection have continued to be reported [87, 90, 91].

4. Genetic Diversity of Modern Korean *P. vivax* Isolates

Malarial infection in history often cannot be fully evidenced by the examination of historical documents. This is due to difficulty in accurate diagnosis of ancient malaria cases in history. Actually, the signs and symptoms of ancient malaria patients were often vaguely described in historical literatures by modern clinical medicine standards. In addition, diagnosis of ancient malaria (solely) by the examination of archaeologically obtained skeletal remains is also highly problematic as malaria leaves little traces on bones. In this regard, we note that DNA-based study could be useful for acquiring scientific evidences of specific diseases prevalent in history [27].

DNA analysis of *P. vivax* is generally targeted on the protozoan parasites' surface proteins by which the erythrocyte invasion of the *vivax*-malarial parasite can be triggered [92, 93]. One such surface protein is the *P. vivax* merozoite surface protein (PvMSP), which is abundantly expressed on the merozoites of *vivax* malaria [94]. Duffy-binding protein (PvDBP) is another membrane protein that is also present on the *P. vivax* merozoites and that plays a crucial role in RBC invasion of parasites [92, 93, 95, 96]. As antibodies against these proteins effectively block the invasion of *P. vivax* into human RBC [91], PvMSP and PvDBP are regarded as leading candidates for use in the development of malaria vaccines [91, 97–99] though great genetic diversity among those surface proteins still represent a major obstacle to the vaccine research [4, 91, 97, 100–103].

Since the reemergence of *P. vivax* in South Korea [48, 87], Korean researchers also have aimed to study the genetic traits of *vivax* malaria's PvMSP, PvDBP, circumsporozoite protein (PvCSP), apical membrane antigen-1 (AMA-1), microsatellites sequences, and 18S ribosomal RNA genes [87, 90, 91, 103–108]. Those scientists are indeed eager to analyze the genetic diversity, population structure, and operation of natural selection among Korean *P. vivax* isolates, as the outcomes would doubtlessly be useful for understanding the nature of the *P. vivax* population in South Korea [90, 91, 97]. In general, the genetic diversity of *P. vivax* is higher than that of *P. falciparum*, suggesting that the former has a long, complex, but successful evolutionary history of adaptation [4, 109–112]. However, when *P. vivax* reemerged in South Korea, the isolates of the years 1993 to 2000 were genetically closely related, meaning that its genetic diversity was very low at the initial stage of its reintroduction [113]. Since 2001, the reemergent malaria population in South Korea has become more heterogeneous, showing increased genetic diversity and a more complex population structure [87, 89, 97, 108]. The results clearly indicate that some genotypes that were not found before 2000 eventually migrated into South Korea at a much later date, as accompanied by outbreeding between different genotypes [90].

Ju et al. [91] also reported that a phylogenetic analysis based on PvDBP sequences showed 3 different clusters (SK-1, SK-2, and SK-3) in Korean *P. vivax* isolates. Among them, SK-3 was a new clade that had not been identified at the early phase of reemergence in the same Korean isolates [106] but later became a more prevalent group than either SK-1 or

TABLE 3: Archaeological information on mummy samples with liver obtained during autopsy.

Number	Mummy	Estimated Date	Sex	Date of excavation (YYYY.MM)
1	Cheongdo	1642 ^a	Male	2014.10.
2	Andong	18C ^b	Male	2013.01.
3	Dalsung	16C-17C ^c	Female	2014.05.
4	Hwasung	18C ^c	Male	2012.12.
5	Gangneung	1622 ^a	Male	2007.11.
6	Hadong2	Late 16-early 17C ^a	Female	2009.06
7	Kunkook	Joseon period ^c	Female	Unknown
8	Mungyeong	1647 ^d	Female	2010.04.

^aHistorical documentation. ^bCarbon dating. ^cArchaeological evidence. ^dTree ring.

benefits, however, the overall genetic trends of vivax malaria, especially concerning its evolutionary history, have not yet been revealed by the simple genomic assay of modern isolates. In fact, the investigation of *P. vivax* using present-day DNA extracts from modern Korean isolates often leads to confusion as to vivax malaria's origin and dispersal [28]. To overcome this drawback, we must conduct aDNA analyses on various human samples obtained from archaeological sites in Korea to analyze the genetic origin and phylogenetic history of malaria more accurately and comprehensively.

The significance of aDNA analysis to any derived understanding of the evolutionary history of malaria recently has been demonstrated by Gelabert et al. [28]. By way of aDNA analysis on 70-year-old microscopic slides of blood from malaria-infected people in Spain, they were able to successfully reconstruct the mtDNA sequence of the now-eradicated European *P. vivax* malaria. Moreover, as it was proven to be related to the most common present-day American *P. vivax* haplotype, the authors were able to confirm that vivax malaria entered the Americas by post-Columbian contact with Europeans [28]. In this way, aDNA assay of ancient human remains can be used for finding the missing links in the origin and spread of ancient malaria.

In aDNA analysis, the types of specimens to choose are very crucial to the research's success. To select the specimens ideal for aDNA assay purposes, the life-cycle of the vivax-malarial parasite must be considered. In brief, when malarial sporozoites are inoculated into human hosts, some of them migrate to the liver wherein they invade the hepatic parenchymal cells [4]. While some sporozoites can maintain the dormant state there, they can be further differentiated into merozoites and released into the bloodstream [4]. As seen in vivax malaria's life-cycle, the liver is the place where the final preerythrocytic phase takes place [45]. In this regard, Joseon Dynasty (1392-1910 CE) mummy's livers might be significant to our project. For the past 10 years, scientists and archaeologists in South Korea have been involved in interdisciplinary work on well-preserved mummies discovered in Joseon Dynasty tombs [117-123]. The livers that could be used for aDNA analysis were obtained from mummies by en bloc resection during autopsy (Table 3) [117].

Nevertheless, as the number of malaria sporozoites at liver stage might be actually very small, we should also consider alternate specimens for our aDNA analysis. In this regard, we note that a small amount (less than 1g) of spongy bones inside vertebrae (possibly containing hemopoietic cell remains) was chosen commonly as specimens for aDNA analysis of malaria; and in another case, first or second molars have been selected for *Plasmodium* aDNA analysis [27]. Many future studies on ancient malarial genomes will proceed with these specimens of Korean mummies or skeletons.

6. Conclusion

With respect to human samples obtained from archaeological sites, scientific techniques can be done to reveal whether the individual had suffered from malaria in his lifetime or to obtain phylogenetic information of its ancient genome. As the previous studies on ancient malaria have focused mainly on specimens from Egypt and Europe, however, the current information so far obtained carries a serious geographical bias. More extensive geographic samplings and assays are thus needed in order to obtain a more comprehensive demographic evolutionary history of malaria.

Like the other continents, Asia is a region wherein malarial infection has been epidemic in history. Nonetheless, very little has been done in the way of relevant paleopathological studies on ancient malaria. We thus reviewed the history of malaria in Korea and attempted to derive scientific clues to the evolution of *P. vivax* there and elsewhere in Asia. To those ends, we first examined the historical-documentary evidence of ancient malarial outbreaks in Joseon society and found that malarial epidemics were in fact not unusual in pre-20th-century Korea. We detected changes in the host-vector-pathogen relationship, which probably affected the proliferation of the mosquito vector and indeed the prevalence of ancient malaria in Joseon society. We also noted, in our review of genomic studies on *P. vivax*, substantial geographic differentiation of vivax-malarial DNA between different continents and even neighboring countries. Many scientific studies on the history of malaria will be done with ancient specimens in Korea and Asia, pending the permission of the relevant medical-ethics review boards.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Dong Hoon Shin and Min Seo contributed equally to this study as first authors.

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