Coll. Antropol. **38** (2014) 2: 749–754
Case report

Skeletal Remains from Late Roman Period: »As Old as Diocletian's Palace«

Davorka Sutlović[1,2], Jelena Ljubković[1], Angela Stipišić[3], Zlatka Knezović[3], Irena Drmić-Hofman[1,4] and Šimun Andjelinović[1,5]

[1] University of Split, School of Medicine, University Hospital Center Split, Department of Pathology and Forensic Medicine, Split, Croatia
[2] University of Split, School of Medicine, Department of Forensic Medicine, Split, Croatia
[3] Public Health Institute of Split-Dalmatian County, Split, Croatia
[4] University of Split, School of Medicine, Department of Medical Chemistry and Biochemistry, Split, Croatia
[5] University of Split, School of Medicine, Department of Pathology, Split, Croatia

A B S T R A C T

In 2000, human skeletal remains were discovered in Split (Croatia). As archaeologists confirmed, it was an ancient skeleton accompanied by ceramics and bracelet characteristic for late Roman period whose possible violent death was excluded. The bone sample was radiocarbon dated by AMS to 1750 years. DNA was successfully extracted from the bone sample and subsequently typed using mt DNA and STR systems. The metal content was determined by atomic absorption spectrometry (AAS) in flame mode. Mercury concentration was determined by direct consecutive measures taken with a mercury analyzer. According to our results, we consider that the bones could belong to the one of the last citizens of the Diocletian's Palace.

***Key words**: lead, late Roman period, ancient bone, mtDNA, elemental status, anthropology*

Case History

Aspalathos was the name of the ancient Greek residence from which modern Split grew. It later became Spalatum under Roman rule and existed as a small settlement until Emperor Diocletian built his luxurious palace there around 300 AD[1]. In the years after his death, during the fall of Roman Empire, expelled Roman emperors made Diocletian's Palace their resort away from home. When Avars and Slavs conquered Salona (now Solin) in 614 AD, its displaced citizens fled to Diocletian's Palace for shelter, began to live inside its walls and set about altering the palace to suit their own housing. Now Split is the second largest city in Croatia, with just under 200,000 inhabitants, and is the largest city on the Adriatic coast (Figure 1).

Individual skeletal remains have been found accidentally in the clay ground, at the northern part of Split (Figure 2). Archeologists confirmed male skeleton, age 30–40, lying on the back in the pit. Forensic examiner was asked to give the answer about the possible violent cause of death, since the skeleton had the metal ring (Figure 3) on the right hand. The ring on the right hand actually was a bracelet, made of copper, with open ends, that characterized aristocracy decoration from assumed time period. The expected maximum stature height, measured by the length of femur, was 167 cm (Trotter and Gleser estimation formulae). Estimated time since death was 1760±80 years (14C method).

Since ancient DNA is an important tool for diverse disciplines, such as anthropology and archaeology, ancient bones and teeth are by far most abundant type of samples available for ancient DNA analysis. The molecular biology techniques provide researchers with the possibility to extract ancient DNA from different sources, even after many thousands of years in order to elucidate genetic relations between species as well as time of their origin and migration routes throughout the history.

Determination of heavy metals' concentration in human remains is well recognized and broadly used technique. Numerous authors previously described how presence

Received for publication June 15, 2012

of different chemical elements could help in reaching conclusions about nutrition habits of ancient populations. Studies based on ancient human remains demonstrate that anthropogenic exposures to metals are reflected in bone and teeth samples and therefore validate in past and future use of ancient bones and teeth as a valuable tool to assess trace element exposure profiles of early societies[2]. In order to determine concentration values of different chemical elements in this skeletal remain's bone samples and the level of environmental pollution in the past, compared with today's pollution, we analyzed above described skeletal remains and the burial site soil.

Materials and Methods

Sample preparation

After anthropological analysis, bone samples were properly labeled and collected for further analysis. First, femur sample was taken for radiochemical analysis (14C method). Other bone samples were washed in deionized water and mechanically cleaned with a plastic brush to remove all possible contaminations present on outer bone surface. A part of a dense cortical bone was cut into pieces for DNA analysis and metal content analysis. Samples were transferred using plastic forceps into the clean vials filled with 5% sodium hypochlorite and shaken occasionally to remove residual impurities. The samples were then acid leached (in 5% nitric acid; HNO_3) to minimize the influence of post mortem contamination, washed 3 times in deionized water and dried at room temperature[3]. Bone pieces were crushed into powder using razor blades and stored in sterile polypropylene tubes at –20°C until analyzed.

DNA analysis

DNA extractions were performed according to Alonso A. et al[4]. The quantification assay was performed using Quantifiler Human assay (Applied Biosystems, Foster City, CA, USA) according to the manufacture's instructions[5].

Autosomal STR analysis

Amplifications were performed with the AmpFlSTR Profiler Plus Amplification Kit (Applied Biosystems, Foster City, CA 94404, USA) according to the manufacturers' instructions[6]. This kit allows simultaneous amplification of eight autosomal STR loci (D3S1358, D8S1179, D5S818, vWA, D21S11, D13S317, FGA, D7S820 and D18S51) and the amelogenin locus (determining the individual's sex). The PCR products were analyzed on ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Mitochondrial DNA analysis

Mitochondrial DNA analyses were performed on the hypervariable region 1 of the mitochondrial DNA control region. This region was divided into two subregions and amplified with overlapping primer sets L15989/H16239 and L16190/H16410[7]. Amplification products were checked on a 1% agarose gel and cloned using TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. Screening of white recombinant colonies was accomplished by PCR and clones with inserts of the expected size were identified by agarose gel electrophoresis. After purification of these PCR products with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), a volume of 1.5 mL was cycle-sequenced following the BigDye Terminator kit v3.1 (Applied Biosystems) supplier's instructions. The sequence was determined using an Applied BioSystems 3130 DNA sequencer. The obtained mtDNA sequence of the skeletal remains were compared to the Cambridge Referal Sequence and assigned to the appropriate haplogroup[8].

Metal content

Reagents used for the extraction and measurement like standard metal solutions (1 mg/L), were Suprapur quality (Merck, Darmstadt, Germany). The conductivity of deionised water used in the experiment was 0.06 μS/cm. Standard solutions were prepared in range of expected concentration values.

Sample digestion

After drying to a constant weight, the sample was soaked in 6 mL 65% HNO_3 over night, washed in deionized water and dried at room temperature. Approximately 0.5 g of the sample was wet-washed in 65% nitric acid and hydrogen peroxide in the Teflon-TFM vessels. The sample with added reagents was left to stand in open tubes at room temperature overnight. Vial was then sealed into the digestion bomb and the automated (temperature regulated) microwave digestion (CEM, USA Model Mars 5–2004 with 1600 W power) protocol was initiated[9]. The microwave program was accomplished in two steps as follows:

Step 1 (a) ramp to 125°C; (b) 15-min to reach preset pressure of 200 psi (1 psi= 6895 Pa); (c) 20-min to hold preset pressure of 200 psi.

Step 2 (a) ramp to 150°C; (b) 10-min to reach preset pressure of 300 psi; (c) 20-min to hold preset pressure of 300 psi. The digestion took about 70 minutes and cooling another 30 minutes. Digested sample was diluted to 50 mL with deionized water. The same analysis was repeated 3 times using samples from different parts of the same skeleton.

Metal determination

Element concentrations of manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), calcium (Ca) and strontium (Sr) were measured with an atomic absorption spectrometer (flame AAS, Analytik Jena AAS Vario 6, Germany) in flame mode[10]. The analysis of lead (Pb) and cadmium (Cd) were performed using Graphite Furnace. Atomic Absorption Spectrometry Concentrations of Fe, Mn, Cu and Zn were determined using Flame Atomic Absorption

Spectrometry with flame C_2H_2/Air. Ca and Sr were determined using Flame Atomic Absorption Spectrometry with flame C_2H_2/N_2O_2 in the same instrument. In each measurement Deuterium background correction was used. Mercury (Hg) concentration was determined by three direct consecutive measured by mercury analyzer[11].

Results

The results of analyses are summarized in Tables 1–3. As carbon analyses revealed, late Roman period skeletal remains were 1760±80 years old (Figure 4, Table 1).

TABLE 1.
THE RESULTS OF ALLELE DETERMINATIONS FROM 3 ANCIENT BONE SAMPLES

Genetic markers	Sample 1	Sample 2	Sample 3
Amelogenin	X;Y	X;Y	X;Y
D3S1358	16;17	16;17	16;17
D8S1179	16	16	16
D5S818	20;23	20;23	20;23
vWA	6;7	6;7;8	6;7
D21S11	8;9	8;9	8;9
D13S317	10	10	10
FGA	10;13	10;13	10;13
D7S820	8;13	8;12;13	8;13
D18S51	7;9	7;9	7;9

It is important to highlight that concentration values of Pb from described skeletal remains greatly exceeded the Pb concentration values when compared with medieval bone samples as well as in surrounding soil (Tables 2 and 3). It is also significance to show that Fe and Hg concentration values were 4 and 2.8 times higher than in modern bone samples.

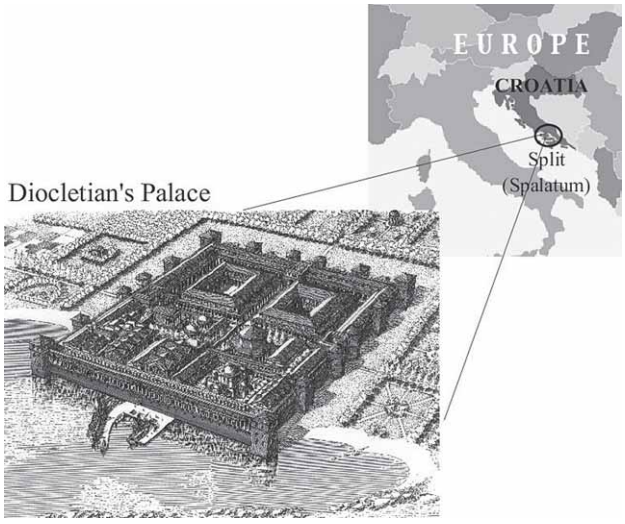

Fig. 1. Geographical location of Croatia Republic, 1700 years old city Split and Diocletian's Palace.

Discussion

In the year of 2000, human skeletal remains have been found at the northern part of Split (Figure 2). The ring found on its right hand was a bracelet, made of copper, with open ends, characteristic decoration for aristocracy from late Roman period (Figure 3). Analysis of carbon C14 showed that the estimated time since death was 1760±80 years. In that period, both men and women wore items of jewellery including rings, bracelets, necklaces and earrings made of different supplies such as stones, glass and metals forming exclusive Roman ornaments. Anthropological measurements and sampling were done at the Department of Forensic Medicine, University Hospital Center Split and School of Medicine, University of Split.

TABLE 2.
METAL CONCENTRATIONS (mg/kg EXCEPT FOR Ca %) IN BONE AND SOIL SAMPLES DETERMINED BY AAS MEASUREMENTS

Sample	Dry weight (%)	Pb (mg/kg)	Cd (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Ca (%)	Sr (mg/kg)	Hg (mg/kg)
Soil sample	96.22	2.89	0.457	173	12755	14.1	52.2	7.67	423	0.0477
Late Roman bone	96.74	19.5	0.019	4.57	104	3.11	106.0	33.97	466	0.0183

TABLE 3.
METAL CONCENTRATIONS (mg/kg EXCEPT FOR Ca %) IN BONES FROM NAKLICE BURIAL SITE AND MODERN BONES SAMPLE DETERMINED BY AAS MEASUREMENTS.

Sample	Dry weight (%)	Pb (mg/kg)	Cd (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Ca (%)	Sr (mg/kg)	Hg (mg/kg)
Bones from Naklice burial site	95.25	2.98	0.03	111.85	1393.29	6.44	143.26	31.16	401.81	0.04
Modern bones samples	93.86	5.40	0.16	5.57	26.01	1.46	102.89	24.73	166.20	0.007

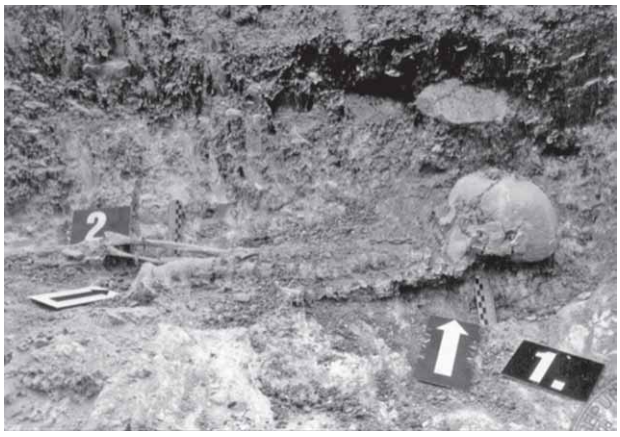

Fig. 2. Skeletal remain found in the clay ground, at the northern part of Split.

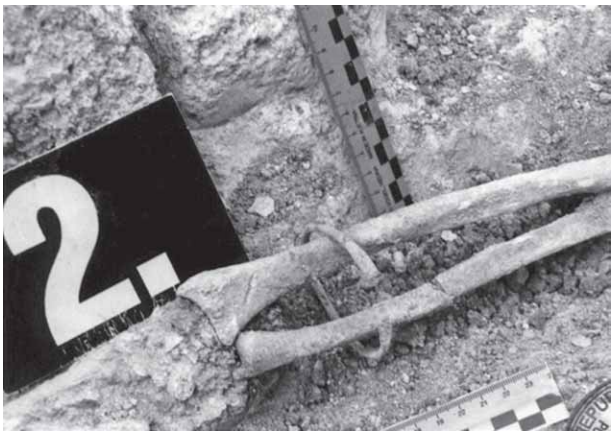

Fig. 3. The metal ring on the right skeletal remains hand, with open ends, which characterized aristocracy.

Archaeological and forensic tests showed that the skeletal remains belonged to the male person which was subsequently confirmed by nuclear DNA analysis using Profiler Plus Amplification Kit (Table 1). It contains specific primers for amplification of amelogenin gene thus allowing sex determination since the X chromosome gene, AMELX, gives rise to a 106 bp amplification product (amplicon) and the Y chromosome gene, AMELY, a 112 bp amplicon[12].

In the bone samples of the the skeletal remains described above, we analyzed the content of Pb, Cd, Mn, Fe, Cu, Zn, Ca, Sr and Hg. Table 1 shows the average concentration values of different elements from the skeletal remains and from the soil sample collected near the skeleton. Table 3 shows the average concentration values of different elements of 16 individuals from five archaeological excavation localities found at Naklice early medieval graveyard (Southern Croatia) and 32 Croatian individuals from modern period[13].

Human exposure to heavy metals present in environment can occur simultaneously from various sources. Possible exposure routes are ingestion of metals through consumption of food and beverages containing those elements, inhalation of atmospheric aerosols or some other routes of contamination and intoxication[14–16]. Correlation analysis leads us to consider the fact that the presence of studied metals in the analyzed bone sample could be the consequence of intoxication[17–20]. In the effort to distinguish eventual sample contamination, we analyzed the soil from the burial site. The limestone soil of burial site had a neutral to mildly alkaline pH (7.88). That kind of soil can strongly binds heavy metals. Therefore, it may slightly reduce the possibility of their accumulation in the sample.

When comparing the results of heavy metals' concentrations of late Roman period skeletal remains and the results obtained by the analysis of Naklice burial site bone samples, it is obvious that observed concentration values of Pb from late Roman period skeletal remains greatly (6.5 times) exceeded the concentration values of these early medieval bone samples while concentration of Mn (25 times) and Fe (13.4 times) were significantly lower.

Concentration value of Mn was 1.2 times lower in late Roman skeleton than this heavy metal's concentration value found in modern bones. Concentration of Sr was similar as observed from Naklice graveyard bone samples but 2.8 times higher than in the modern bone samples. Fe and Hg concentration values were 4 and 2.8 times higher than in the modern bone samples. It could probably be due to the environmental contributors at archaeological site and content of Fe and Hg in the soil environment.

Taking into account the present-day environmental pollution, we expected lower Pb concentration value in the studied skeletal remains than in the modern bone samples, but it was 3.6 times higher than in the modern bone samples.

According to the applicable standard, Drasch found that in the late Roman epoch, in the Middle Age, lead concentration value was 41–47% of today observed value[21].

High concentration value of lead could be due to lifestyle, industrial pollution or nutrition. It is well known and confirmed that skeletal remains analyzed in this paper dates from 3 centuries AD which brings into question the possible cause of this high concentration value obtained for this metal. Seeking an answer, we have come to the conclusion that lead accumulated in this skeletal remains due to lifestyle and use of ceramic pottery with lead glazes.

De la Villa et al. described the results obtained by analyzing late Roman amphora fragments taken from the Museo Municipal of Ceuta, Spain and raw material from different clay seams[22]. The results showed high lead concentration, achieving a value up to 183 mg/kg. Amphorae were used among members of the wealthier social classes especially during time period from which described skeletal remains belongs.

From archaeological sources, it is known that lead glazes were used from the second millennium BC and

were made of non-clay minerals containing lead. One of the most significant periods for glazed ceramic production was that between the 2nd and 3rd centuries AD. Ceramics were characterized by a bi-color glaze green and yellow-honey. These ceramics are very abundant in the Italic Peninsula, southern Gaul and the Iberian Peninsula. During the later Roman Empire, glazed ceramics underwent further major changes. They were transformed from luxury into household ceramics, all decorations were removed and a thicker glaze was used.

Conclusion

According to the results of all performed (C14 carbon, STR, mtDNA and metal content) analysis, completed with archaeological findings based upon jewelry and ceramic items, we consider that the 1750-year-old male skeletal remains found in Split (Fig. 4) belongs to a nobleman who lived at the same time as Emperor Diocletian. His HV1 mtDNA sequence belongs to mitochondrial haplogroup H, as it didn't show any other significant changes that would suggest otherwise (compared with Cambridge Referral Sequence). This is one of the oldest and most abundant European mt DNA haplogroups that includes members of many European royal families. High concentration of lead discovered in his bones is the result of eating from bowl dish made of lead glazes used by wealthier members of society. Finally, we conclude that these skeletal remains refer to one of the last citizen of Emperor Diocletion's Palace before the Palace was inhabited by refugees of surrounding areas.

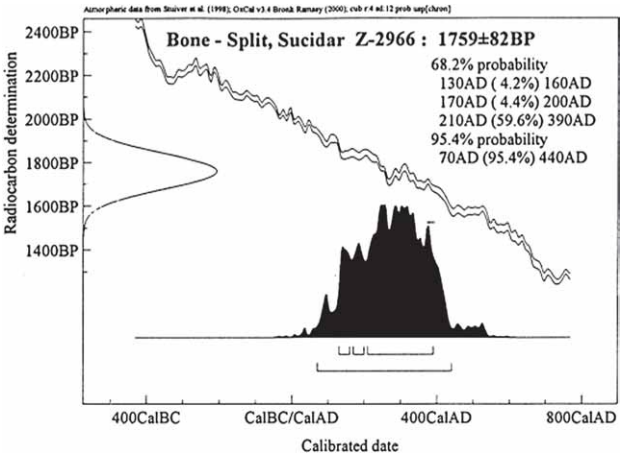

Fig. 4. Result of radiocarbon bone analysis.

Acknowledgements

We would especially like to thank Ms Boja Režić and Ms Ana Samardžić for excellent technical assistance, and Marko Ljubković, PhD MD, for proofreading and corrections of the manuscript.

This study was supported by the Ministry of Science, Education and Sports of the Republic of Croatia, »Heavy metals in human remains from Klis and Bribir ancient counties«; grant No. 216-2160800-0655 and »DNA analysis of an early medieval population from South Croatia« grant No. 141-2160800-0333.

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D. Sutlović

University of Split, School of Medicine, University Hospital Center Split, Department of Pathology and Forensic Medicine, Spinčićeva 1, 21000 Split, Croatia
e-mail: dsutlov@kbsplit.hr

KOSTUR IZ KASNOG RIMSKOG DOBA: STAR KOLIKO I DIOKLECIJANOVA PALAČA

S A Ž E T A K

U 2000.-oj godini u Splitu (Hrvatska) otkriven je kosturni ostatak. Prema pronađenoj keramici i narukvici, arheolozi su potvrdiili da je pronađeni kostur karakterističan za kraj rimskog razdoblja. Nasilna smrt bila je isključena. Metodom radioaktivnog ugljika analiziran je uzorak kosti te je određena starost od oko 1750 godina. Uspješno je izolirana DNA i uspješno je umnožena koristeći mtDNA i STR sustav. Sadržaj metala određen je plamenom atomskom apsorpcijskom spektrometrijom (AAS). Koncentracija žive utvrđena je direktnom metodom koristeći analizator žive. Prema našim rezultatima, smatramo da kosturni ostaci mogu pripadati jednom od posljednjih stanovnika Dioklecijanove palače.