Evaluation of the DNA extraction method from ancient animal bones

Jurgita Baublienė[1], Linas Daugnora[1], Ilona Miceikienė[2]

[1] *Department of Anatomy and Histology*
[2]*Department of Animal Breeding and Genetics Lithuanian Veterinary Academy,*
Tilžės str. 18,
LT-3022, Kaunas, Lithuania
E-mail: jurga.baubliene@lva.lt

Different materials were used to answer the questions connected with the past, but the most abundant and surviving material is bone. However, the yield of aDNA is strongly related to the state of preservation, which is affected by burial conditions.

The aim of our study was to extract from different animal bones good quality aDNA suitable for further investigation. Totally, 20 bones of cattle, horse, sheep, goat and aurochs were tested. DNA yield was retrieved using the Richards et al. (1995) extraction method, followed by a purification recommended by the Yang et al. (1998) protocol. The recovered colour of the supernatant was estimated. The quality of aDNA was evaluated according to PCR (polymerase chain reaction) results. Three samples from 11 cattle and auroch bone samples were amplified.

Key words: ancient DNA, bone, polymerase chain reaction

INTRODUCTION

The use of ancient DNA (aDNA) in different fields is becoming increasingly common. The results of the investigations have been applied in physical anthropology, archaeology, evolutionary biology and forensic science. The data can reveal the sex of individuals, the indigenous or migrant populations, the history of animal and plant domestication, and phylogenetic relationship between modern and extinct species (Burger et al., 1999; Stone, 2000; Wayne, Leonard 1999). Different archaeological material was used for aDNA extraction. In the nineties research primarily was aimed at successfully extracting ancient DNA from soft tissues, bone, tooth, coprolites, seeds and other plant materials. The discovery that DNA can be recovered from ancient bones (Hummel Hagelberg et al., 1989; Herrmann and Hummel, 1994) has offered new possibilities for the study of past populations, because bone has a lot of advantages against other excavated materials.

The main advantage of the bone is that it is most commonly surviving and abundant skeletal material in the archaeological sites. When material is well preserved it is very easy to collect, thus many museums throughout the world can contain extensive osteological collections. The main difference of hard tissue preservation state may be the result of the hydroxyapatite content in the first place (Herrmann and Hummel, 1994; MacHugh et al., 1999, 2000). Roughly 2/3 of a bone is inorganic and 1/3 organic. The inorganic fraction consists mainly of hydroxyapatite and a lesser extent of fluorapatite. Secondly, bone is a material lacking in liquids and enzymes, therefore cells within the bone can be expected to suffer less from autolytic process and probably to be better protected against diagenetic influences than cells of any other tissue. Finally, teeth and bones are less affected by natural contamination (microorganisms, fungis), and contemporary contaminations are likely to be removed prior to extraction.

Typically, the quality and quantity of DNA isolated from an individual is better from bone than from soft tissue. Also, there is some evidence that DNA survives better and longer fragments can be amplified from bone than from soft tissue samples (Richards et al., 1995; Stone, 2000; Götherstrom, 2001). The main reason for this is bone structure. Hydroxyapatite forms the framework through ion exchange with the surroundings. The crystal is constantly altering and becoming more crystalline. In 2001, Götherstrom found a high correlation between crystallinity and DNA preservation. There is also a correlation between DNA preservation and collagen preservation, though not as strong as that between DNA and hydroxyapatite (Götherstrom, 2001).

When an organism dies, its DNA normally becomes degraded by endogenous nucleases. Under

fortunate circumstances, such as rapid desiccation, low temperatures or high salt concentrations, nucleases can themselves become destroyed or inactivated before all nucleic acids are reduced to mononucleotides (Hofreiter et al., 2001; MacHugh et al., 2000). Assuming physiological salt concentrations, neutral pH and a temperature of 15 °C, it would take about 100,000 years for hydrolytic damage to destroy all DNA that could be reasonably retrieved (Hoxreiter et al., 2001). Thus, genetic material survival and quality seems to be related rather to the preservation state of the bone, which is not directly correlated with age but depends on the burial environment (Herrmann and Hummel, 1994; Poinar et al., 1996; Richards et al., 1995).

MATERIALS AND METHODS

Archeological bone preparation and DNA extraction. We used cattle (*Bos taurus*), horse (*Equus cabalus*), sheep (*Ovis aries*), goat (*Capra hircus*) and auroch (*Bos primigenius*) bones excavated in different excavation sites for aDNA extraction. Totally, 20 bones (horn, tooth, ulna, tibia) were tested. Cattle bones were found in the Klaipėda castle dating to the 14th–16th centuries, and in the Vilnius tale castle dating to the 16th century; horse bones were found in Marvelė (9th–12th centuries), sheep and goat bones were found in Kernavė (14th–16th centuries) and auroch bones in Šventoji (5600/5400–4400/4300 B. P., Middle Neolithic).

The samples were prepared for aDNA extraction by removing any surface contamination (cleaning each bone surface with vacuum). A flame-sterilized hacksaw cut up bone into pieces, and bone powder was collected by two powdering methods – by drilling and by grinding, depending on bone preservation status. Different amounts of bone powder yielded from sample to sample ranged from 0.5 to 14.0 grams depending on the bone powdering method. For each aDNA extraction, 300 mg to 1.5 g of bone powder was used, following a modified version of the procedure detailed by Richards et al. (1995) and Yang et al. (1998). Each sample of bone powder was incubated at 55 °C for 12 h with 0.5 M EDTA pH 8.0, proteinase K 200 μg/ml, 0.5% Sarcosyl and 1.0 M Tris–HCl pH 8.0. Then the temperature was lowered to 37 °C and incubation was continued for the next 24 h. The extraction solution was centrifuged with a Heraeus centrifuge at 5.000 rpm for 10 min and again spun in a microcentrifuge 13,000 rpm, for further 2 min. After centrifugation the supernatant was transferred to a 10 ml tube with a Nalgene filter and centrifuged. The recovered supernatant was mixed with five volumes of QIAquick PB buffer and centrifuged. The flow-through was discarded and the process was repeated until all of the extract passed through the column. DNA was washed by adding QIAquick PE buffer and centrifuging. The flowthrough was discarded and additional TE buffer and centrifuging then eluted DNA from the column. The final extraction volume varied between 80 and 120 μl.

To avoid contamination and incorrect interpretation of results two blank extractions and one control extraction (sample known to work) were included, also one negative control (C-labelled equined or porcine bone from the same site) was made. The laboratory equipment and materials were UV-irradiated.

DNA amplification. Extracts were amplified in 18 μl reactions comprising 10 × PCR buffer, 50 mM $MgCl_2$, 2 mM dNTP, 4 μM each primer, 0.2 μl platinum enzyme, 8.8 μl ddH_2O, 2.0 μl DNA sample. A 2 min denaturation step at 94 °C was followed by 40 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (30 s), and then a 4 min final extension step at 72 °C.

The most variable segment of the bovine mtDNA (mitochondrial DNA) control region was targeted for PCR amplification. This segment had been previously identified and characterized through mtDNA sequence variation surveys in a range of cattle populations (Bradley et al., 1996). Primers were designed using the reference GenBank bovine mitochondrial genome sequences: $AN2_{FOR}$ – $ANI1_{REV}$ and $AN1_{FOR}$ – $AN3_{REV.}$ The PCR primers and their location and orientation in the reference sequence are: $AN2_{FOR}$ (16.022–16.041), $AN1_{REV}$ (16.178–16.159), $AN1_{FOR}$ (16.159–16.178), and $AN3_{REV}$ (16.334–16.314).

To check for PCR inhibition, 4 μl of each sample was taken and added to 1 μ of spike (ancient sample known to work).

After amplification, the PCR products were visualized and evaluated on 1.5% agarose gel with 100 bp ladder.

RESULTS AND DISCUSSION

The skeletal material was excavated from Medieval and middle Neolithic settlements. The geologic conditions of the excavation sites were cool and dry, and the skeletal material was in uniformly excellent condition. Bones were chosen confirming the excellent preservation of morphological conditions. The skeletal material was generally of a light brown colour similar to fresh bone, with a little of dark discoloration found in buried bones subjected to frequent perfusion by groundwater and accompanying compounds. Many naturally occurring compounds found in soil and groundwater, such as humid ac-

ids, tannins, iron, cobalt and other materials, can become incorporated into archeological material, co-extracted and inhibit PCR reactions.

In Table, details of the archaeological bone powdering method and aDNA supernatant quality after first and second extraction steps are shown. The method of powdering was chosen depending on the degree of bone preservation. Tough bone samples were prepared by drilling, while fragile bones were ground with grinders. The recovered bone powder yield was sufficient for the subsequent reactions. The recovered supernatant after the first extraction step was stained mainly with a brown colour, caused by humid acids in the soil and were thought to be at least partially responsible for the inhibitors present in archaeological skeletal remains. When the second extraction was made, most of the samples were found suitable for the subsequent PCR reaction. The colour intensity of the supernatant varied in different extraction samples from pale to brown.

The relative DNA content recovered from the supernatant can be established by determining the rate of a successful polymerase chain reaction (PCR) (Höss et al., 1993; Hummel et al., 1992; MacHugh et al., 1993). The quantity and quality of the results can vary, depending on the prevailing environmental factor or storage conditions. In our research, successful aDNA extraction from different animal bones was evaluated according to PCR results. Three samples out of 11 cattle and auroch bone samples were successful and a PCR product was obtained. The success rate of approximately 27% is not very sufficient, because it stands far away from the results obtained by Richards et al. (1995). Richards et al. (1995) suggest that more than 50% of skeletal remains from the past 2000 years are likely to contain amplifiable endogenous aDNA. Two successful cattle bone samples were from the Klaipėda castle excavation site and one from the Vilnius tale castle site. The excavation places were dated to the Medieval Age, but not all samples were from the medieval period; auroch bones were found in a middle-Neolith settlement. From the past 2000 years came 9 samples, and a success rate in them was 34%. To check the recovered aDNA and inhibitor yield, a sample known to work in the PCR reaction was used. For testing, a auroch sample was used. The PCR reaction was made with eight samples, which failed in the first amplification reaction. Four samples out of eight yielded amplifiable aDNA, and in 4 samples there was no amplifiable DNA. Successful PCR reactions show that the extracted aDNA supernatant contained too much inhibitor, while the aDNA yield was not enough for the reaction.

The main problem during our work was an insufficient aDNA supernatant yield and quality. Lying in the ground, bones were affected by environmental conditions, such us salt concentration, temperature, groundwater balance, which are directly correlated with recovered genetic material. So during the extraction step all undamaged and not restricted aDNA is recovered for the PCR reaction. Also, in the recovered supernatant many naturally occurring compounds from the soil and groundwater can be found, because they are incorporated into archaeological material through hundreds of years. In the result of such inhibition, the amplification reactions fail. Therefore the methods are constantly

Table. **Archaeological bone powdering method and extraction supernatant quality**

Sample code*	Bone powdering method	Recovered bone powder (g)	Amount of powder used in purification (mg)	Supernatant color intensity after first extraction	Supernatant color intensity after second extraction
A-1	drill	7.20	316	brown	golden
A-2	drill	1.20	260	brown	brown
A-3	drill	0.80	318	brown	brown
A-4	drill	0.80	285	brown	brown
A-5	drill	1.70	279	brown	brown
A-6	drill	0.50	273	brown	golden
A-7	drill	0.50	282	brown	golden
A-8	grind	4.58	454	yellow	pale
H-2	grind	1.00	268	brown	golden
H-4	drill	8.00	393	golden	golden
H-6	grind	9.70	312	golden	yellow
AO-3	grind	11.46	420	brown	golden
B-2	drill	1.40	380	brown	brown
B-3	drill	2.00	586	black	brown

* A – cattle (*Bos taurus*) bone samples; H – horse (*Equus caballus*) bone samples; AO – goat (*Ovis aries*) or sheep (*Capra hircus*) bone samples; B – aurochs (*Bos primigenius*) bone samples

improving, and new ideas are generated while working with excavated material.

Another problem is extraneous contamination, which can change PCR amplification results and their interpretation. An example could be DNA of micro-organisms, which were incorporated into the sample from the ground and from people who were working with archaeological material and could leave their DNA on it. To avoid wrong results, specific primers are chosen for the PCR, and sequencing reactions are carried out.

To get satistactory results, all extraction and purification steps must be repeated accurately and carefully. The environment must be clean and isolated from the other laboratory rooms where experiments are made with new DNA.

References

1. Bradley D. G., MacHugh D. E., Cunningham P., Loftus R. Mitochondrial diversity and the origins of African and European cattle. *Proceedings of the National Academy of Sciences of the USA.* 1996. Vol. 93. No. 10. P. 5131–5135.
2. Burger J., Hummel S., Herrmann B., Henke W. DNA preservation: a microsatellite-DNA study on ancient skeletal remains. *Electrophoresis.* 1999. Vol. 20. No. 8. P. 1722–1728.
3. Götherstrom A. Acquired and inherited prestige? Molecular studies of family structures and local horses in Central Svealand during Early Medieval period. *Theses and Papers in Scientific Archaeology 4.* Archaeological Research Laboratory, Stockholm University, 2001.
4. Hagelberg E., Sykes B., Hedges R. Ancient bone DNA amplified. *Nature.* 1989. Vol. 342. No. 6249. P. 485.
5. Herrmann B., Hummel S. *Ancient DNA. Recovery and analysis of genetic material from paleontological, archeological, museum, medical, and forensic specimens.* New York: Springer-Verlag, 1994. P. 1–12, 59–67, 205–210.
6. Hofreiter M., Serre D., Poinar N., Kuch M., Pääbo S. Ancient DNA. *Nature Reviews.* 2001. Vol. 2. No. 5. P. 353–359.
7. Höss M., Pääbo S. DNA extraction from Pleistocene bones by silica-based purification method. *Nucleic Acids Research.* 1993. Vol. 21. No. 16. P. 3913–3914.
8. Hummel S., Nordsiek G., Herrmann B. Improved efficiency in amplification of ancient DNA and its sequence analysis. *Naturwissenschaften.* 1992. Bd. 79. N 8. P. 359–360.
9. MacHugh D. E., Troy C. S., McCormick F., Olsaker I., Eythorsdottir E., Bradley D. Early medieval cattle remains from a Scandinavian settlement Dublin: genetic analysis and comparison with extant breeds. *Philosophical Transactions of the Royal Society of London.* 1999. Vol. 354. No. 1379. P. 99–109.
10. MacHugh D. E., Edwards C. J., Bailey J. F., Bancroft D. R., Bradley D. G. The extraction and analysis of ancient DNA from bone and teeth: a survey of current methodologies. *Ancient Biomolecules.* 2000. Vol. 3. No. 2. P. 81–103.
11. Poinar H. N., Höss M., Bada J. L., Pääbo S. Amino acids racemization and the preservation of ancient DNA. *Science.* 1996. Vol. 272, No. 5263. P. 864–866.
12. Richards M. B., Sykes B. C., Hedges M. Authenticating DNA extracted from ancient skeletal remains. *Journal of Archaeological Sciences.* 1995. Vol. 22. No. 2. P. 291–299.
13. Stone A. C. *Biological anthropology of the human skeleton.* Chapter 13: Ancient DNA from skeletal remains, Wiley-Liss; A John Wiley & Sons, Inc., 2000. P. 351–371.
14. Yang D. Y., Eng B., Waye J. S., Dudar J. C., Saunders S. R. Technical note: improved DNA extraction from ancient bones using silica-based spins columns. *American Journal of Physical Anthropology.* 1998. Vol. 105. No. 4. P. 539–543.
15. Wayne R. K., Leonard J. A. Full of sound and fury: the recent history of ancient DNA. *Annual Review of Ecology and Systematics.* 1999. Vol. 30. No. 1. P. 457–477.

Jurgita Baublienė, Linas Daugnora, Ilona Miceikienė

DNR IŠSKYRIMO IŠ SENOVĖS GYVULIŲ KAULŲ METODO ĮVERTINIMAS

S a n t r a u k a

Per pastaruosius du dešimtmečius sėkmingai pritaikius polimerazinę ciklinę reakciją (PCR) tapo įmanoma išskirti senovinę DNR (sDNR) iš archeologinės medžiagos. Darbas su senovine DNR labai išpopuliarėjo, nes pasitelkus šias molekules galima atsakyti į daugelį su praeitimi susijusių klausimų. Viena populiariausių tyrimuose naudojamų medžiagų tapo kaulai, kurie dėl unikalios struktūros gali ilgai sveiki išsilaikyti žemėje.

Mūsų darbo tikslas buvo išskirti sDNR ekstraktą, tinkamą tolesniems tyrimams. Šiam tikslui įgyvendinti buvo naudojami galvijų, arklių, avių, ožkų ir taurų (*Bos primigenius*) kaulai. Tirta 20 kaulų. Pirmiausia kaulai buvo smulkinami vienu iš dviejų (trupinant ar gręžiant) pasirinktu kaulų smulkinimo būdu. Vėliau buvo skiriamas sDNR tirpalas. sDNR tirpalo kokybė buvo vertinama pagal gautą supernatanto spalvą ir kiekį. Vienuolika kaulų pavyzdžių buvo amplifikuojami panaudojant variabilią mitochondrinės DNR seką koduojančius pradmenis. Pirmos amplifikacijos metu pavyko 3 pavyzdžiai iš 11. Atlikus pakartotiną amplifikaciją su žinomu ir patikrintu sDNR pavyzdžiu, gauti 4 teigiami rezultatai iš aštuonių. Dar keturiuose rezultatuose neaptikta sDNR.

Raktažodžiai: senovinė DNR, kaulas, polimerazinė ciklinė reakcija