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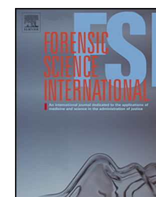

Technical Note

Technical note: Efficient removal of a PCR inhibitory agent (vivianite) found on excavated bones

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A B S T R A C T

During a rescue excavation in October 2011, archaeologists discovered a mass grave with 10 individuals. The skeletons should belong to victims of the battle of Reichenberg between the Austrian and Prussian armies on April 21, 1757. Several bones of the skeletons were covered with a blue colored encrustation. Initial DNA analysis failed due to strong inhibition. Chemical analysis of the bluish encrustation indicated the presence of the iron phosphate mineral *vivianite* ($Fe_3(PO_4)_2{\cdot}(H_2O)_8$). This technical note describes a novel procedure for the removal of this inhibitory substance.

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1. Introduction

During a rescue excavation in October 2011, archaeologists discovered a mass grave with 10 male individuals at the Nerudovo square of Liberec, Northern Bohemia, which was dated to the year 1757 (see Fig. 1). The skeletons should belong to victims of the battle of Reichenberg (fought on April 21, 1757) between the Austrian and Prussian armies. The dating of the burial site was based on the historical records and also on the artifacts found with the skeletons (e.g. the military buttons). The battle was part of the Seven Years' War. Several bones of the skeletons were covered with a blue colored encrustation (see Fig. 2). Chemical analysis of the bluish encrustation indicated the presence of the iron phosphate mineral *vivianite* ($Fe_3(PO_4)_2{\cdot}(H_2O)_8$). Vivianite requires specific conditions for its formation—sources of iron, phosphate, and water, as well as low levels of oxygen and sulfide. Microbial activity is also thought to play a role in *vivianite* formation [1,2]. The presence of vivianite on skeletal remains was reported in several previous studies, including those from ancient bisons [3], "Brienzi" man [4], and Ötzi [5], but also on the skeletal remains of Americans missing in action in Vietnam [6]. Iron compounds may have an inhibitory effect on PCR [7,8], and thus, it is necessary to employ special procedures for the removal of those inhibitory agents [9,10]. Procedures employing the addition of the chelating compound EDTA [11] or sample dilution [12] are not suitable for samples with minute amounts of DNA extractable from aged skeletal remains [13]. This technical note describes the procedure for the removal of *vivianite* prior to PCR.

2. Material and methods

2.1. Chemical analysis

The chemical analysis of the bluish encrustation was performed using X-ray fluorescence (XRF) analysis [14] and Fourier transform infrared spectroscopy [15].

2.2. DNA analysis

The bone powder from the skeletal remains (femurs) was prepared according to the procedure described previously [16]. The grinding procedure was slightly changed as we used 6770 FREEZER/MILL-SPEX Sample Prep (SPEX SamplePrep, Metuchen,

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Fig. 1. Excavation site with 10 male skeletons at the Nerudovo square of Liberec, North Bohemia.

NJ, USA) instead of a grinding mill (Warring, Torrington, CT, USA). All bone samples used for the analysis were partially covered with a bluish encrustation.

2.3. Pre-extraction cleaning

We placed 0.5 g of bone powder in 50 ml tubes, covered them with 20 ml 0.1 M $NaHCO_3$ and incubated them for 1 h at room temperature with occasional mixing (flipping of the tube). The tube was centrifuged for 4 min at 2000 rpm. The top aqueous phase was aspirated. The upper solid phase of a grey-bluish color and mud consistency was removed. The sample for DNA extraction was taken from the middle or lower phase. Collected phases of cleaned bone powder are shown in Fig. 3.

2.4. DNA extraction

The extraction was performed in triplicate (60 mg per extraction) from the same phase (see above). DNA from pre-treated bone powder was extracted using the PrepFiler®BTA Forensic DNA Extraction Kit (Life Technologies, USA). We used a recommended protocol in which we prolonged the initial incubation (220 μl PrepFiler BTA Lysis Buffer + 7 μl proteinase K + 3 μl 1 M DTT) from 2 h to 18 h (overnight). The resulting 3 eluates (50 μl Elution Buffer) from the same phase aliquots were pooled together and further processed as one sample.

2.5. Post-extraction cleaning

The DNA extract was further cleaned with Zymo Research OneStep™ PCR Inhibitor Removal Kit (ZymoResearch, USA) and

Fig. 2. An example of blue colored encrustation on bones recovered from the excavation site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

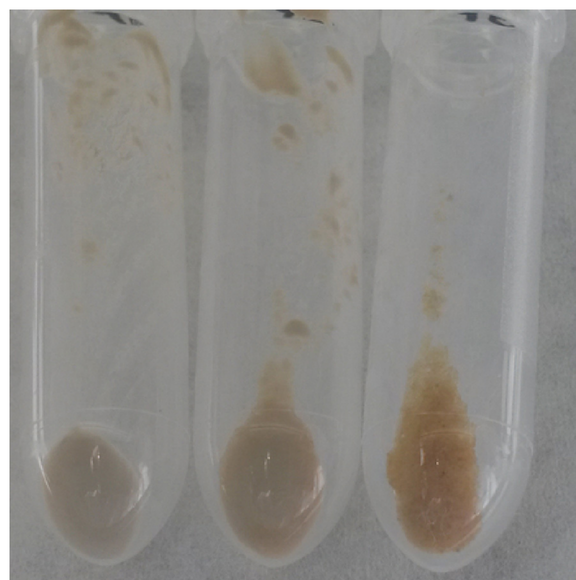

Fig. 3. Collected phases of the bone sample (left tube = upper phase, middle tube = middle phase, right tube = lower phase). Middle and lower phase was used for DNA extraction. Upper phase was discarded.

concentrated to 100 μl on a SpeedVac vacuum concentrator (Eppendorf, Germany). The subsequent dialysis step was performed on a 25 mm MF-Millipore membrane composed of mixed cellulose with a 0.025 μm pore size (Merck Millipore, USA). We used distilled water as the dialysis medium. The dialysis took 45–60 min. The product of dialysis was concentrated on a SpeedVac vacuum concentrator (Eppendorf, Germany) to 7.5 μl, and the whole volume was used for the subsequent PCR.

The scheme of the pre-extraction cleaning, DNA extraction, and post-extraction cleaning steps is presented in Fig. 4.

2.6. DNA quantitation

Extracted DNA was quantified by real-time PCR (SYBR Green assay, target sequence ALU transposable element, amplicon size 63 bp) using the 4N6 Quant kit (Forenzni DNA servis, Czech Republic) on a MasterCycler ep realplex S instrument (Eppendorf, Germany).

2.7. PCR amplification

The PCR amplification was performed using the AmpFLSTR Yfiler PCR Amplification Kit (Life Technologies, USA). The PCR reaction was as follows: total volume of 25 μl, 2.5 μl STR boost (Biomatrica, USA), and a sample volume of 7.5 μl. We used the recommended protocol with increased number of cycles to 32 cycles (MasterCyclerR ep gradient S thermocycler, Eppendorf, Germany).

2.8. Post PCR cleaning

The PCR product was cleaned and concentrated using Amplicon Rx™ (Independent Forensics, USA).

2.9. Fragment analysis

Amplified STR fragments were separated on an ABI PRISM 310 Genetic Analyzer (Life Technologies, USA) under standard conditions. Samples were injected (5 kV injections) for 10 s. The

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