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Forensic DNA analysis in the identification of human remains in mass graves

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Routine techniques are often insufficient to address the identification of human remains in mass graves. The major complicating factors include delayed exhumation, commingling of skeletal remains, lack of ante-mortem information and attempts to conceal evidence of criminal activity. We have elaborated a study on two mass graves that contained partly fragmented commingled remains from the ethnic conflicts that took place in 2001 in the Republic of Macedonia. By using DNA typing of autosomal and Y-chromosome short tandem repeat (STR) markers and by making comparisons with samples from parents and siblings we identified all of the victims.

Key words: DNA, autosomal and Y-chromosomal STR, decomposed bodies.

INTRODUCTION

There are a variety of forensic techniques in use today that can be applied to the identification of human remains. The choice depends upon the circumstances and the condition of the remains to be examined. Commonly used but not particularly reliable techniques include visual comparisons of special features, e.g. specific scars, tattoos, etc. Definitive techniques (fingerprint comparisons, dental comparisons) largely depend on the actual structural preservation, as well as the availability of pre-mortem records Alonso et al. (2001). During times of acute social conflicts, major atrocities, mass disasters or terrorist attacks with multiple casualties, identification of remains in common burial sites becomes more complex and is hampered by a lack of pre-mortem data Definmis-Gojanovic et al. (1995) and Holland et al. (2003). Nowadays, these complexities and the public expectations impose a burden on the application of DNA technology Andgelinovic et al. (2005). In any particular mass disaster, there are many inter-related factors and circumstances that may challenge the final DNA identification goal. Those include, but are not limited to, the number of victims, the mechanism of destruction and the extent of the remains' fragmentation, the rate of degradation of the

DNA, the accessibility of samples to be collected, and the availability of DNA reference samples Alonso et al. (2005). Short sequences and a high degree of polymorphism make the short tandem repeats (STR) method practical and amenable to amplification by PCR. Information about the genetic diversity of the male-specific portion of the human Y-chromosome, especially the Y-chromosomal short tandem repeats (Y-STRs), has grown considerably over the past decade Jobling et al. (1997) and Roewer et al. (1992). The Y - STRs have recently been established for routine casework in paternity testing, particularly in deficiency cases, as well as in forensic stain analysis Hiroyoshi et al. (2002), Prinz et al. (1997), Roewer et al. (1996) and Shneider et al. (1998). More than 14 Y - specific STR markers are known to provide simple, sensitive, reproducible and reliable markers for the identification of male individuals Kayser et al. (1997).

CASE REPORTS

Two mass graves were excavated that contained commingled complete and partial remains. These were suspected to be the result of atrocities stemming from the ethnic conflict in the Republic of Macedonia in 2001. We performed autopsies on the exhumed victims and forensic DNA analyses using autosomal and Y - chromosome STR haplotyping of the samples, obtained from the victims'

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remains and from potential surviving relatives. The evidentiary materials were collected following the INTERPOL recommendations for identification procedures, to guarantee sample preservation for DNA analyses and to document the chain of custody of the DNA samples Interpol (1998). The professional multidisciplinary team included an investigative judge, a prosecutor, forensic pathologists, a forensic anthropologist, a biologist, a team of crime laboratory technicians from the Ministry of Interior (MI) and observers from the International Criminal Tribunal for the former Yugoslavia (ICTY), Organization for Security and Co-operation in Europe (OSCE), EU monitoring missions and PROXIMA (EU Police Mission). Due to the sensitive nature of the ethnic conflict that generated these cases (ethnic Macedonians were buried in the first mass grave; ethnic Albanians were buried in the second), which caused a lack of trust in the state institutions and an increased need of objectivity, the DNA analyses of the same samples were performed and reaffirmed at 1) the Institute of Forensic Medicine and Criminology, Skopje, Republic of Macedonia, 2) the DNA Laboratory of the Macedonian Academy of Sciences and Arts, and 3) the International Society for Missing Persons (ISPM) DNA Laboratory, Sarajevo, Bosnia. The major complicating factors such as delayed exhumation, commingling of skeletal remains, lack of ante-mortem information and attempts to conceal evidence of criminal activity, rendered it impossible to make identification only by applying forensic autopsy. Thence, the identification had to be performed with a forensic DNA analysis of bones. One of the factors that hindered the identification process in the mass grave in Neprosteno was the excavation of the grave by the perpetrators of the criminal act in order to remove and hide the bodies.

MATERIALS AND METHODS

Pursuant to the INTERPOL recommendations, ante-mortem information was obtained from the families that were likely to be connected with the cases. Standard medico-legal procedures were applied in the autopsies. We estimated the age, sex and stature of the victims by the usual methods and formulae (Bass, 1995; Halund et al., 1997). The anthropological assessment of the skeletal remains, the pathological changes and any evidence of trauma were documented in detail. Furthermore, comparisons of the tentative ante-mortem dental records with the documented post-mortem dental status were carried out. The whole process of the exhumations was recorded by one fixed and two mobile video cameras and the evidentiary photographs were taken using two conventional 35mm cameras and two digital cameras.

Mass grave found near the village of Neprosteno

The exhumation process from the mass grave detected at this site took place between November 21 and November 25, 2001. According to the operative findings of the investigative authorities (competent court, public prosecutor and Ministry of Interior), approximately 6 months prior to the discovery, a group of ethnic Albanians had removed the victims' remains from their original

burial sites and had disposed of the remains in this mass grave, in an attempt to conceal their acts. This mass grave had overall dimensions of 9 × 4 × 16 m and it contained multiple commingled remains at different levels (Figure 1).

The remains recovered from the grave site during this exhumation consisted of two upper halves of the skeletonized remains, three femora, two pelvises, and bones from a foot. Applying the standard medico-legal methodology, we were able to establish that the commingled skeletonized remains belonged to four male individuals. A gunshot wound was detected in the femur of one set of the remains, whereas in the other human remains there were no injuries. The autopsies could not determine the cause of death, while positive identification of the remains could not be accomplished by anthropological assessments.

Mass grave found at Jama location

The exhumation of the remains detected at this location took place on May 17, 2004. Four decomposed, clothed bodies were recovered. By using standard anthropological methods, we established that the remains belonged to 4 male individuals. All 4 victims displayed multiple mechanical injuries caused by blunt-force trauma due to a fall, as well as gunshot wounds. The mechanical injuries in all 4 bodies were post-mortem. At that point, the unavailability of ante-mortem information precluded a definitive identification.

Sources of the samples for the DNA analyses

Samples of the bones, teeth and hair were obtained for the genomic DNA analyses from the remains from both mass graves. Blood samples and buccal swabs were obtained from the alleged families of the missing individuals. There were 28 relatives, from 9 families, sampled in reference to the remains recovered from the Neprosteno mass grave. There were 27 relatives, from 8 families, sampled in reference to the remains recovered from the Jama mass grave. The standard Whitman FTA cards were used for the storage of the blood samples.

DNA extraction from the reference blood samples

The DNA samples were extracted from the whole blood via QiaAmp DNA MiniKit (QIAGEN, Hilden, Germany) following the manufacturer's instructions for the whole blood protocol.

DNA extraction from the bones of the recovered skeletal remains

All the bone surfaces were cleansed of the remnants of soft tissues and soil contaminants, brushed in warm water and mild detergent, rinsed several times in distilled water and air-dried. The bone fragments were washed in a commercial bleach solution and subsequently washed 3 times in de-ionized water and twice in 70% ethanol solution, prior to air-drying for 24 h. The bones were pulverized using Dremel tools. For decalcification, 40 - 45 ml of 0.5 M EDTA at pH 7.5 was added to 3 to 5 grams of bone powder and left in a shaker for 24 h at room temperature. The mixture was centrifuged for 15 min at 2,000 rpm, the supernatant was discarded, and an additional 40 - 45 ml of 0.5 M EDTA added. The procedure was repeated for a total of 3 times. The pellet was rinsed in 16 ml of distilled water and centrifuged for 15 min at 2,000 rpm. The supernatant was discarded and the procedure was repeated 2 more times. Subsequently, 3 ml of extraction buffer (10 mol/l Tris pH 8.0; 100 mol/l NaCl; 50 mol/l EDTA, pH 8.0 and 0.5% sodium dodecyl

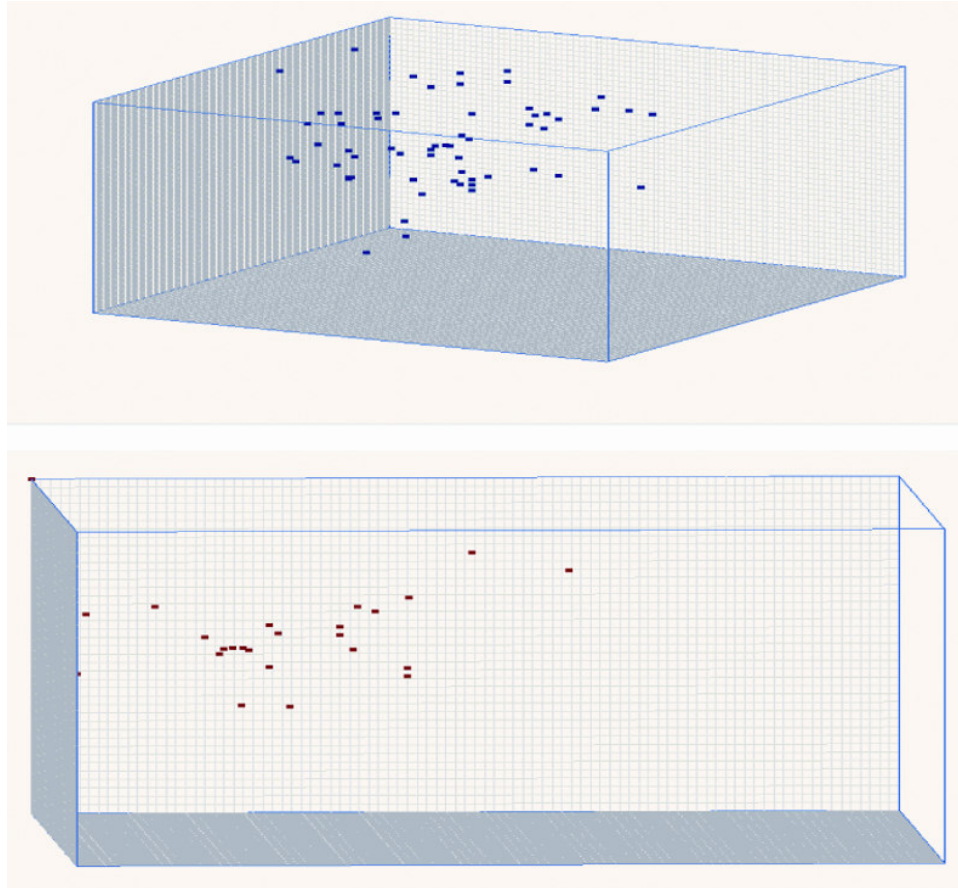


Figure 1. This mass grave had overall dimensions of 16 × 9 × 4 m and contained multiple commingled remains at different levels.

sulphate) and 0.5 ml of 20 mg/ml proteinase K were added to the bone sample. This mixture was incubated at 56°C, for 48 h and DNA was isolated by the organic phenol/chloroform/isoamyl alcohol extraction.

PCR reaction

Multiplex PCR amplification was performed using 1 - 3 ng of genomic DNA according to the manufacturer's protocol for the AmpFISTR Identifier kit, Promega PowerPlex[®]16 kit and PowerPlex[®] Y kit. Amplification was carried out in a 9600 Thermal Cycler (Applied Biosystems). For electrophoresis with AmpFISTR Identifier kit, 1.5 µl of the PCR product was combined with 12 µl of formamide and 0.5 µl of GeneScan 500 LIZ size standard. For electrophoresis with Promega PowerPlex[®]16 and PowerPlex[®] Y kits, 1 µl of the PCR product was combined with 24 µl of formamide and 1 µl of ILS size standard. The detection of PCR products and genotyping were carried out on the ABI PRISM 310 Genetic Analyser (Applied Biosystems) using the ABI PRISM collection software, ABI Prism 310 Data Collection software 3.1.0 and Gene Mapper v 3.2 (Applied Biosystems). The statistical analyses were conducted using DNA View software.

RESULTS AND DISCUSSION

This was the first multidisciplinary investigation of the

mass graves related to the ethnic conflicts in the Republic of Macedonia. In all cases, we obtained a complete profile for autosomal and Y chromosomal STR's and we identified with a probability of 99.99%, the remains as those of persons previously reported missing. Adequate collection, analysis and preservation of the evidence for potential further review by independent experts, in line with the international guidelines, were essential in maintaining the integrity of the identification of the remains in the mass graves. With the standard medico-legal methodology, in both cases of mass graves, it was only possible to establish the number of bodies, the sex, the approximate age and stature, as well as, in some cases, the mechanism of injury.

The method of extraction of DNA used here, where there is degraded DNA material, provides sufficient amounts of DNA for PCR reaction and it has shown a high elimination of PCR inhibitors. The presence of the international experts from ICTY, PROXIMA, OSCE and EU monitoring missions, in the capacity of observers throughout the identification process, had a positive social impact due to the nature of the ethnic conflict, in which the identified victims were of both Macedonian and Albanian ethnicity. We can conclude that definitive identi-

fication of human remains in situations of intentional disposal and concealment becomes impossible if one is to rely only on routine autopsy and anthropological techniques, although these remain an integral part of the process in the ever-expanding multidisciplinary endeavor, which is strengthened by the advanced DNA technology. The information obtained in a multidisciplinary fashion paved the way for judicial proceedings before the Macedonian and the ICTY courts in both cases. Our experience demonstrates the applicability of PCR to human biological material in advanced stages of degradation. The completion of the database, as well as the submission of biological material by the missing persons' relatives, imposed certain difficulties since due to the ethnic conflict both sides were rather hesitant in terms of the state institutions. In order to overcome the forgoing problem, the support of the missions of ICMP, ICTY, PROXIMA, OSCE and EU was very helpful, as they managed to convince the relatives of the missing persons to provide the biological material necessary for the identification. The use of separate and independent institutions (Institute of Forensic Medicine, Criminology and Medical Deontology in Skopje, Republic of Macedonia; Macedonian Academy of Sciences and Arts; ICMP, Sarajevo, Bosnia) was important in the verification and reproducibility of the scientific evidence presented subsequently in Macedonian and ICTY courts because such an approach helped build confidence among citizens from different ethnic backgrounds relative to the professional competence and integrity of the domestic experts and the institutions of the state. Our experience reiterates the multidisciplinary approach as the optimal route in handling these investigative challenges.

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