Identification of Human Decomposed Remains Using the STR Systems: Effect on Typing Results

Pavel L. Ivanov [1, 2], and Maya V.Isaenko [2].
[1] Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia.
[2] Center for Forensic Medical Expertize, Ministry of Health; Moscow, Russia.

This report covers our starting experience on the DNA identification of the post-mortem remains recovered from the battlefields, and illustrates some effects on typing results when STR systems are used for DNA typing of decomposed human remains. This study is primarily concerned with the amplification of degraded DNA samples. Reports of identification by family studies will be presented elsewhere.

BACKGROUND

The Russian government plans efforts to identify deceased military personnel who came from the recent military "law enforcement" mission in Chechnya region. One particular feature of the Chechen war is the very high proportion of MIA's. Of the total 6000 soldiers lost in that war, 1500 were not returned to their families for burial and were declared missing in action. (It is ten times as much as in Afghanistan, where the ratio of battle deaths/MIA's is approximately 1:46). At the same time, hundreds of bodies remain unidentified among more than 4500 reported battle deaths.

It should be noted that the identification of soldiers killed in the line of duty is not an entirely novel concept for the Russian military. However historically, until recently, the success in identifying war dead was not the question of the highest priority for the country, and no special resources and assets have been devoted to this endeavor. Currently, the identification of an unknown soldier is made primarily through dental techniques or bone radiographic analysis. Metal medallions (kind of "dog tags") carried by soldiers are not considered identification since they can be easily switched, but they do serve as a starting point for pulling records.

The Chechen war started 11 December 1994 and ended 31 August 1996. During this period, and then after the war, all the soldiers who died in Chechnya battles - both recently killed and excavated different periods after death - were transported to the specialized mortuary at the Armed Forces Regional North-Caucasus Hospital which is located in the city of Rostov-on-Don. The fallen soldiers are positively identified before their bodies are sent to their families for burial. In all cases when identification is required, forensic examiners and military pathologists at the local Armed Forces forensic laboratory perform a detailed examination of the clothing and belongings of the dead, describe special features, analyze remains to estimate sex and height, and compare premortem dental records. In addition, X-ray comparisons are performed for bone morphology, along with identification by the superimposition of skull and photographic images. The compiled premortem data are compared with the autopsy findings.

In modern warfare, however, the lethal force of missiles can sometimes leave few remains, making it difficult to identify dead soldiers. That has happened in the Chechen war. By the end of 1997, some 500 bodies remained unidentified, being kept in freezers in the Rostov mortuary. The military officials have certified them as unknown soldiers, having decided that it was impossible to identify their remains using traditional methods. Meanwhile, Russian authorities consider it essential to retrieve, identify and bury all of the soldiers killed in this battle; hence, the former battlefields in Chechnya are currently under examination and 500 more unknown remains are expected to be recovered after exhumations in the city of Grozny and other places are completed.

The time has come, and officials recognize the value of DNA testing as a necessary adjunct to traditional identification efforts. In a Memorandum No. 1052 [1] dated August 20, 1997, the Prime Minister of the Russian Federation authorized the Ministry of Defence, Ministry of Interior, and Ministry of Health to establish policies and requirements for identity testing of remains from the Chechen war including possible use of DNA analysis in the identification of the remains. The problem is that there is no comparison DNA on file for the soldiers: none of the thousands of government troops in Checnya have had their DNA analysed or at least collected for identification purposes. Hence, the collection and registration of familial data on missing personnel who had disappeared during the war has been started to assist in the remains identification process, including the search for parents and other close relatives to provide reference material for DNA analysis.

In line with this activity, the military officials requested representative tissue samples to be sent to the DNA laboratory at the Office of the Chief Medical Examiner to see if it could get a DNA profile of the remains. The goal of this study was to evaluate the reliability of individualization, and demonstrate whether the frequently encountered condition of biological decay will adversely affect the DNA profile patterns produced. One of the authors (P.L.I.) has been assigned to collect test DNA specimens and further direct the DNA typing as sort of a validation study.

DNA SOURCES

Samples of long bones, cartilage, tendons, hairs, teeth, muscle and other soft tissues were collected at the time of examination of the unidentified bodies, which are being kept frozen at -15° C at the military mortuary. Excavated up to three years after death, the remains were either completely skeletonized or in moderate or advanced stage of decomposition depending on the postmortem interval. Some had been partially or completely saponified or mummified. Some were scavenged by animals. While the circumstances of burying and excavation varied, in general, the bodies were buried and excavated carelessly; the cause of death was not always apparent. Large group of remains comprised incinerated and extremely damaged and fragmented bodies and body parts of service members who were blown apart by explosives (rockets, artillery shells, grenades, landmines, etc.). Some body fragments were carbonized.

METHODS

Because the use of restriction fragment length polymorphisms (RFLP) is not applicable in cases involving extensive degradation of DNA, we considered identifying war victims by PCR-based DNA typing. A choice was made to explore the feasibility of using amplified fragment length polymorphisms (AmpFLP) and amplified fragment site polymorphisms (AmpFSP). In the latter case we analyzed the HLA DQA1 locus and the five loci of the Amplitype PM system using original Perkin Elmer PCR Amplification and Typing kits [2, 3].

With reference to the AmpFLP tests, in our work we used both VNTRs and STR loci. We used both systems made in-house for VNTR-AmpFLPs and some of STRs [4, 5], and commercially available kits for typing STR loci - *GenePrint*™ STR Systems - produced by Promega Corporation [6]. In addition, we have applied a DNA-based sex test to selected samples by amplifying the X-Y homologous gene amelogenin [7].

In our protocols, we used ethidium bromide or silver staining to detect the presence of amplified products following their separation by native or denaturing polyacrylamide gel electrophoresis containing 7% glycerol. (In our work, the necessity for denaturing polyacrylamide gel electrophoresis was investigated with respect to resolution and accurate allele identification, and compared resolution for STR alleles was obtained using medium formate vertical gels of both types.)

We have evaluated different DNA extraction methods to prepare DNA for amplification. As a rule, both phenol and non-organic extraction methods have been used for the processing of a majority of the samples. Standard organic extraction consists of digest buffer/Proteinase K with phenol/chloroform purification. As an alternative, we used Chelex100 [8]. Both the organic extraction and Chelex procedure were finalized with Centricon-100 wash and concentration; extracts were quantified using the DyNA Quant 200 minifluorometer (Pharmacia).

We routinely amplify 1-50 ng of template DNA in a 25-50ul reaction volume according to the manufacturer's instructions using protocols optimized for GeneAmp PCR System 9600 (Perkin Elmer, USA), TouchDown PCR block (Hybaid, UK), and Cyclotemp 5 Thermal Cycler (STM, Russia).

RESULTS AND DISCUSSIONS

The overall results of this work showed that, under the conditions studied, DNA preparations obtained from different types of test specimens can be sometimes successfully amplified and typed at the HLA DQA1 and PM loci, as well as at a number of VNTR and STR loci. There were many cases in which totally six amplified fragment site polymorphisms (AmpFSPs) including the HLA DQA1 locus and the five loci of the Amplitype PM system, as well as six VNTR-AmpFLP and six-eight STR-AmpFLP profiles were reliably identified. The combination of all these genetic loci analyzed by PCR used to give a relative population frequency of 1 in several billions for the donors of the sample.

The potential success in typing the degraded tissue samples serves to emphasize the importance and usefulness of a PCR/STR amplification approach to identifications, when decomposed or partially incinerated remains are encountered.

However, in many cases AmpFLPs at 16-70-nucleotide VNTR loci are not properly identified in highly degraded DNA from the decaying tissue. Very often the integrity of DNA was altered such that the overall amplification pattern becomes extremely weak or even undetectable. In other cases a danger of false AmpFLP patterns arises. Mostly they are well known false homozygotes, but occasionally

artifact bands of the amplification reaction appeared as an additive to characteristic patterns. These spurious bands were similar to those which are typically generated from good quality DNA when excessive amount of template is added to the reaction, and likely represent misprimed products or single-stranded products or heteroduplexes. Meanwhile, titration of the template input in our case had little effect on the appearance of artifact bands, and in the extreme, reactions simply failed to amplify. This may reflect that large proportions of degraded material in such DNA preparations adversely affect the intrinsic balance between the true and bypass products for the particular amplification reaction. This sometimes make it very difficult to determine the true genotype at a given locus, thus suggesting that given the extent of DNA degradation in the sample, any analysis based upon VNTRs would be limited.

Tetranucleotide STR typing, with amplification products that are much smaller than the material detected with VNTR-AmpFLP analysis, and whose alleles do not differ widely in size, has the advantage of being less sensitive to the use of degraded DNA and do not demonstrate allelic dropout. Although these systems are more effective than VNTR/PCR systems in DNA typing decomposed remains, nevertheless, if the STR locus under investigation has a relatively high molecular weight, the alleles amplification may as well fail due to the lack of sufficient intact target molecules. In turn, in the multiplex systems, the inverse correlation was evident between the efficiency of amplifications from highly degraded DNA and the length of the expected PCR product. In our study we observed this when Promega's CTT triplex PCR amplification system was applied to some bone DNA preparations (Fig. 1): the locus with larger alleles (e.g., CSF1PO) was the first to fail in amplification, whereas the loci with shorter alleles (TP0X and TH01) go next.

Analysis of the amplified DNA in a number of the muscle and bone samples revealed artifact PCR products which contain a significant amount of mass (see Fig. 1, lane 1, 2). In some instances, titration of the template input improved the typing result (Fig. 2), whereas in other cases, similar results were achieved when 5 ng of DNA template was used instead of 50 ng (Fig. 3). The band pattern remained ambiguous: when analyzed, the multiple products resulting from the amplification of degraded genomic DNA fell within allelic size range specific to the locus, and consequently, these products created a problem when assigning allelic type.

There are several possible causes for the appearance of the extra bands. In many cases for the products which fall within a narrow allelic size range this can be explained by the phenomenon known as repeat slippage [9], resulting in the display of extra bands below the authentic alleles (Fig. 4, lane 1). Generally, the presence of repeat slippage products, often referred to as stutter bands, are more strongly correlated with particular locus than with other amplification conditions. With the exception of the vWF locus [10], the STR loci used in this study normally generate amplification products with very little stutter. However, nonspecific PCR product bands were observed at many loci in degraded samples, and therefore in this instance they are not peculiarities of the vWF system. Occasionally, stutter bands and specific allelic products observed in the particular degraded sample looked indistinguishable, thus making the overall pattern very similar to allelic ladder marker and inappropriate for scoring alleles at a locus (Fig. 4, lane 4, 5).

In few instances, when more than one sample per body was typed, the stutter bands were not observed in some tissue specimens, but appeared in conjunction with characteristic amplified product in another tissue, which could have been in a more advanced stage of decomposition (Fig. 5). In particular, the example in Fig. 5 may reflect the fact that DNA in deep muscle is more susceptible to degradation than DNA in tooth matrix or in dried skin, and that a correlation exists between the amount of spurious product produced in an amplification reaction and the extent of DNA degradation.

Specific degradation products that appear as above mentioned locus-specific allelic ladder were also seen when different samples from one body were analyzed (Fig. 6). Multiple fragments are produced within the allelic size range with four bases difference in length, which create a ladder-like pattern. These ladders mask or even completely absorb the true genotype allelles. It is noteworthy, that the component fragments of such a ladder seem to not be typical repeat slippage products. They are located not only below authentic alleles, but above as well. These artifact bands contain approximately equal amount of mass, and are the only amplified products observed.

Examples of ladder-like electropherograms from a number of degraded samples tested with STR mono-systems and multiplex CTT are shown in Fig.7 and 8.

We have tested possible causes for the appearance of such an artifact, and showed that such a ladder can be obtained experimentally. An experiment has been designed where 50 ng of control high molecular weight DNA was chemically cleaved with 0.3N NaOH in a boiling water bath, neutralized with 2.5N HCl, and than amplified at known heterozygous STR locus. By monitoring the extent of chemical degradation of input DNA, a spectrum of reproducible patterns was seen ranging from clear

characteristic genotype in conjunction with faint ladder like background, then typical allelic ladder containing 7-10 fragments with no dominating genotype alleles, and further to "no product" result.

We evaluated a selection of control samples for degradation. The molecular weight of test DNA was conventionally evaluated by electrophoresis in denaturing polyacrylamide gel following by silver staining. In the analysis, human genomic DNA which had been boiled in 0.3N alkali for 15 minutes, 30 minutes, and 50 minutes appears as an asymmetric smear of DNA with mass peak area sized at 400, 160, and 40bp, respectively, relative to an appropriate molecular weight marker (not shown).

When amplified at heterozygous vWA31/A locus [10] (Fig. 9) the two DNA preparations ("15-min" and "30-min") both generate 8-component ladder; the third ("50-min") reaction failed to amplify, most probably due to excessive degradation of the DNA. Note that for the "15-min" sample the true alleles are still distinguishable whereas for "30-min" sample only smaller alleles can be defined. The effectiveness of amplification from both degraded samples was similar and approximately tenfold lower compared to the intact DNA preparation.

Sex tests were conducted on these DNA samples. In all three cases a positive gender result was obtained; the amount of PCR product amplified from the "15-min" and "30-min" DNA was the same, whereas that from the "50-min" sample was at least 10 times less (not shown).

One more result is shown in Figure 10, where "15-" "20-", and "25-min" human tissue culture strain K562 DNA preparations were amplified at heterozygous vWFII locus [12]. Again, a full 7-component set of known allelic fragments appears on the gel, and eventually, no allelic type can be assigned.

It is apparent from these results that the phenomenon of degradation artifact ladder cannot be ascribed simply to repeat slippage. Given our observations, it is reasonable to conclude that it may be caused by other mechanisms. At a given locus, the more degraded the sample, the lower the concentration of available template DNA and thus the lower the final amount of characteristic PCR product produced from a degraded sample. At the same time, degradative breaks in the DNA expose short fragments which, under condition of profound degradation of template DNA are compared in length with the locus size range. These degraded DNA short fragments may statistically contain only part of the locus possessing one primer binding site - either forward or reverse. Under condition of their relatively high concentration, they could govern cycle synthesis of single stranded lengths of locus-specific DNA which, in turn, could in some instances anneal to each other at complementary tandem repeat stretches in a 4 bp phasing manner. In the shortage of normal amplifiable templates, this could eventually lead to amplification of spurious product of ladder-like pattern.

This highly schematic model is just in part consistent with what has been demonstrated in this study, and needs to be further detailed.

Anyway, the appearance of such ladder-like artifact seems to be an indication of little hope for successful typing at a given locus due to excessive degradation of the DNA. Although such an issue is disappointing, nevertheless, there is one positive moment: one can use a phenomenon of degradation artifact described in this study as a cheap and effective way to produce allelic markers for at least some STR/PCR systems.

REFERENCES

1. Memorandum No. 1052 (20 Aug 1997), *The Government of Russian Federation*; **Access Number 20004429**.DOC.

2. *AmpliType User Guide, Version 2*, (1993) The Perkin-Elmer Corporation.

3. *AmpliType PM & PM+DQA1 PCR Amplification and Typing Kits*, (1995) Roche Molecular Systems, Inc.

4. Efremov I.A., (1996) Study on the Allelic polymorphisms of Micro- and Minisatellite Loci in Human Genome by PCR Amplification Approach. *Ph.D. Thesis*, Moscow, Russia.

5. Yefremov I.A., Zayatz M.V., Ivanov P.L. Expert Evaluation of Molecular Genetic Individualizing Systems Based on Tetranucleotide Tandem Repeats HUMvWFII and D6S366.

6. *GenePrint*™ STR Systems (Silver Stain Detection), (1998) Promega Corporation

7. Mannucci A., Sullivan K.M., Ivanov P.L., Gill P., (1994) Forensic Application of a Rapid and Quantitative DNA Sex Test by Amplification of X-Y Homologous Gene Amelogenin. *Int. J. Leg. Med.*, **106**:190-195.

8. Walsh P., Metzger D., Higuchi R., (1991) Chelex 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material. *BioTechniques*, **10**:506-513.

9. Schlotterer C., Tautz D., (1992) Slippage Synthesis of Simple Sequence DNA. *Nucleic Acids Res.*, **20**:211-215.

10. Walsh P.S., Fildes N.J., Reynolds R., (1996) Sequence Analysis and Characterization of Stutter Products at the Tetranucleotide Repeat Locus vWA. *Nucleic Acids Res.* **24**:2807-2812.

11. Kimpton C.P., Walton A., Gill P., (1992) A Further Tetranucleotide Repeat Polymorphism in the vWA gene. *Hum. Mol. Genet.*, **1**:287.

12. Ploos van Amstel H.K., Reitsma P.H., (1990) Tetranucleotide Repeat Polymorphism in the vWF Gene. *Nucleic Acids Res.*, **18**:4957.

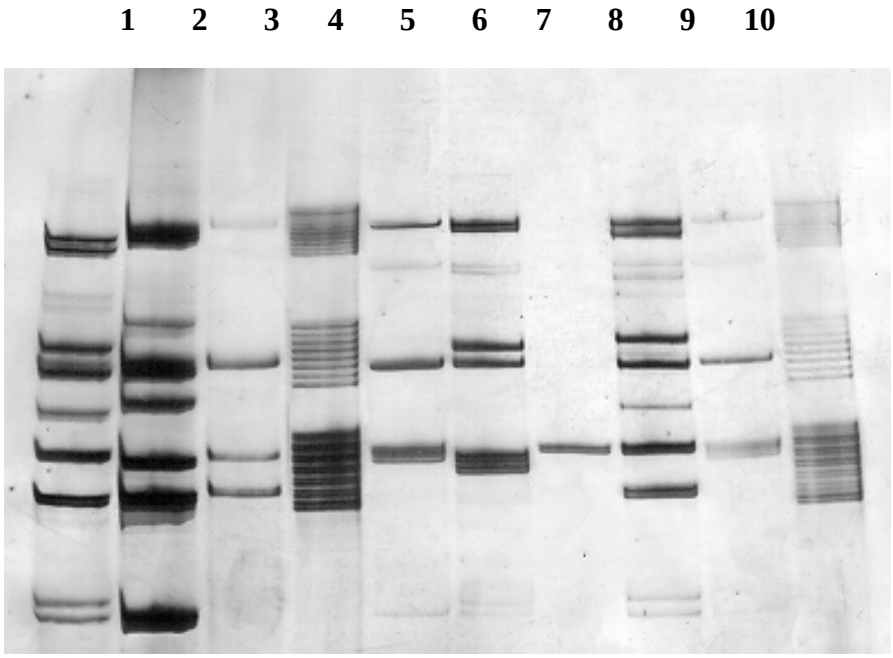

Figure 1. *GenePrint* ™ Multiplex CTT amplification system applied to DNA preparations from exhumed bones: the locus with larger alleles HUMCSF1PO is the first to fail in amplification from highly degraded DNA (Lane 3, 7, 9); the locus with shorter alleles, HUMTPOX, is the next candidate (Lane 7). Lanes 4 and 10 - CTT allelic ladder.

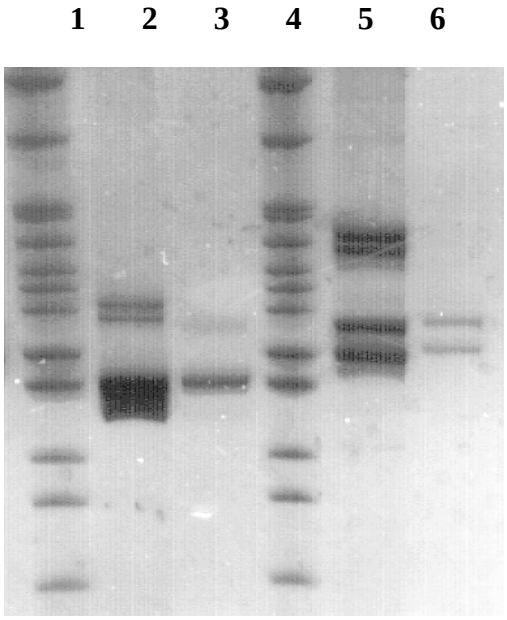

Figure 2. Optimization of the reaction for the DNA input: 50 ng and 5 ng of template DNA in HUMvWA31/A system (Lanes 2, 3); 50 ng and 5 ng of template DNA in HUMTH01 system (Lanes 2, 3); Lanes 1 and 4 - molecular weight marker.

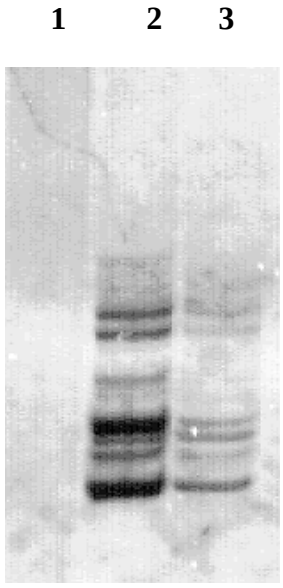

Figure 3. Non-effective titration of the reaction for the DNA input: 50 ng and 5 ng of template DNA in HUMTH01 system (Lanes 2, 3); Lane 1 - negative control.

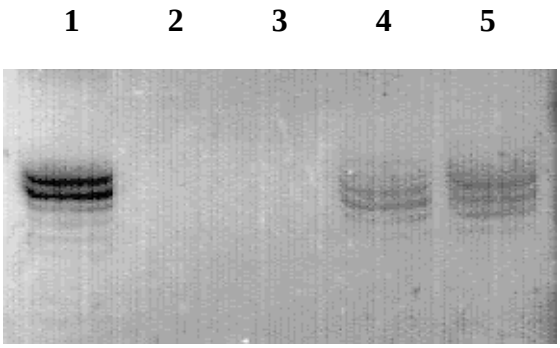

Figure 4. Stutter artifacts (repeat slippage) in HUMvWA31/A system.

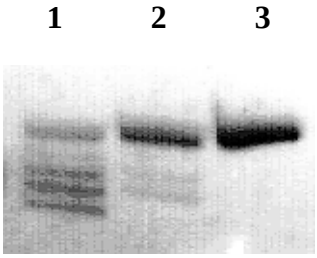

Figure 5. Different tissue samples from one decomposed body typed with HUMF13B system: muscle, skin, tooth (Lanes 1, 2, 3, correspondingly).

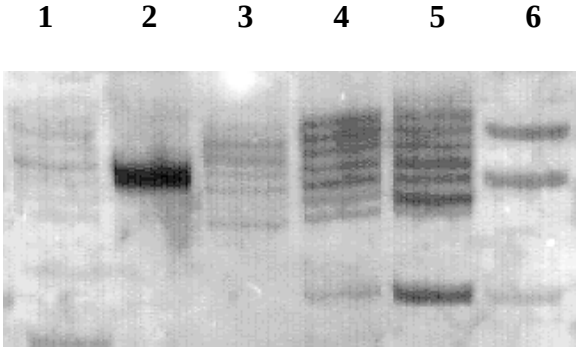

Figure 6. Different tissue samples from one decomposed body typed with HUMvWFII system: hair root, tooth, bone, and the two muscle specimens (Lanes 1-5, correspondingly). Lane 6 - heterozygous positive control.

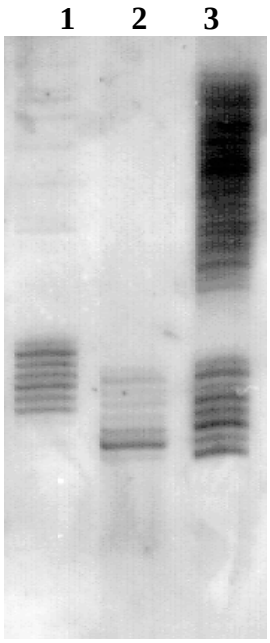

Figure 7. Examples of ladder-like electropherograms from degraded samples (muscle) typed with STR monosystems HUMvWFII and HUMvWA31/A (Lanes 1, 2). Lane 3 - vWA allelic ladder.

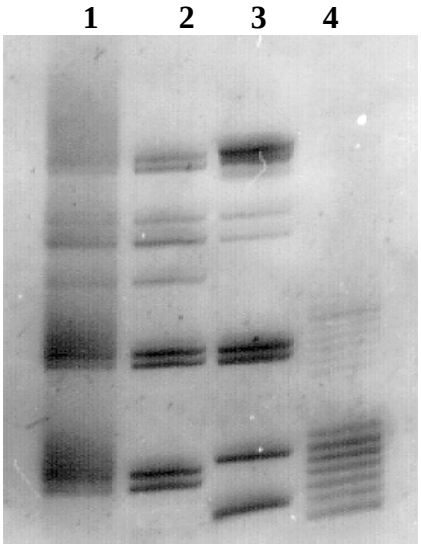

Figure 8. Ladder-like artifact in degraded sample typed with STR multiplex system CTT. Lanes 1-3 - amplification profiles from exhumed bone samples; Lane-4 - amplification profile from decaying muscle.

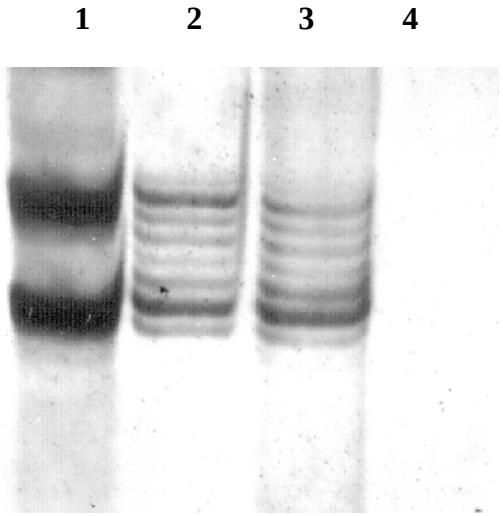

Figure 9. Experimentally testing possible cause for the appearance of the ladder-like artifact with HUMvWA31/A system: effect of chemical degradation of template DNA (see text). Amplification profiles from 50 ng of intact high molecular weight DNA (Lane 1), and from the same DNA which has been treated with alkali for 15 min, 30 min, and 50 min (Lanes 2-4, correspondingly).

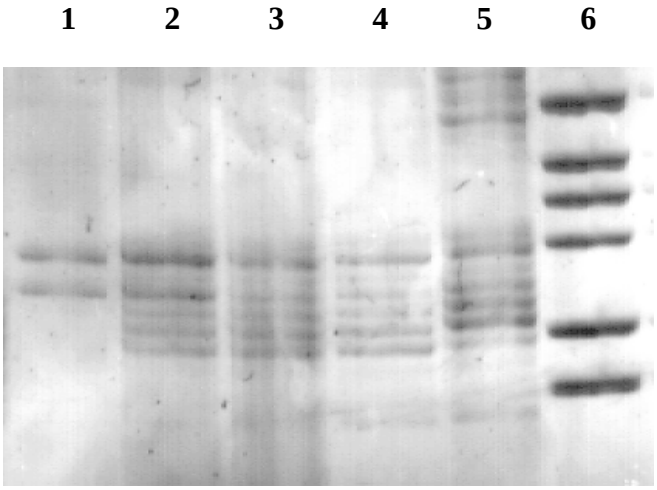

Figure 10. 5 ng of intact high molecular weight DNA (Lane 1), and "15-min", "20-min", and "25-min" human tissue culture strain K562 DNA preparations amplified at heterozygous vWFII locus (Lanes 2-4, correspondingly). Lane 5 - vWFII allelic ladder. Lane 6 - molecular weight marker.