Int J Leg Med (1991) 104:229–233

International Journal of
Legal Medicine

PCR-based typing of DNA extracted from cigarette butts

M. N. Hochmeister[1], B. Budowle[2], J. Jung[2], U. V. Borer[1], C. T. Comey[2], and R. Dirnhofer[1]

[1]Department of Forensic Medicine, Pestalozzistrasse 22, CH-4056 Basel, Switzerland
[2]Forensic Science Research and Training Center, Laboratory Division, FBI Academy, Quantico, VA 22135, USA

Received March 22, 1991 / Received in revised form June 5, 1991

Summary. Limited genetic marker information can be obtained from saliva by typing by conventional serological means. Thus, the application of PCR-based DNA typing methods was investigated as a potential approach for typing genetic markers in saliva. DNA was isolated from 200 cigarettes smoked by 10 different individuals (20 cigarettes per individual) and from 3 cigarette butts recovered from 2 crime scenes (adjudicated cases) using a Chelex 100 extraction procedure. The amount of recovered human DNA was quantified by slot-blot analysis and ranged from approximately <2–160 ng DNA per cigarette butt for the 200 samples, and 8 ng, 50 ng, and 100 ng for the cigarette butts from the adjudicated cases. The DNA was successfully amplified by the polymerase chain reaction (PCR) for the HLA-DQ alpha locus (99 out of 100 samples) as well as for the variable number of tandem repeat (VNTR) locus D1S80 (99 out of 100 samples). Amplification and typing of DNA was successful on all samples recovered from the crime scenes. The results suggest that PCR-based typing of DNA offers a potential method for genetically characterizing traces of saliva on cigarette butts.

Key words: Cigarette butts – Saliva – DNA – Polymerase chain reaction (PCR) – HLA-DQ alpha – D1S80 (pMCT118) – AMP-FLP – human identification

Zusammenfassung. In Anbetracht der limitierten Möglichkeiten der Typisierung von Speichelspuren mittels konventioneller serologischer Methoden wurden für diesen Anwendungsbereich DNA-Typisierungsmethoden, die auf der Polymerase-Chain-Reaction (PCR) basieren, untersucht. Aus 200 Zigaretten, die von 10 verschiedenen Personen geraucht worden waren (20 Zigaretten pro Person), sowie aus 3 von 2 Tatorten stammenden Zigarettenkippen wurde DNA mittels Chelex 100 Methode isoliert und die Menge humaner DNA im Slot-Blot Verfahren bestimmt. Aus den 200 Zigarettenkippen konnten zwischen <2 –160 ng DNA extrahiert werden, aus den von den Tatorten stammenden Zigaretten 8 ng, 50 ng, bzw. 100 ng DNA. Die DNA wurde mittels PCR erfolgreich amplifiziert und typisiert (99 von 100 Zigarettenkippen am HLA-DQ alpha Locus; 99 von 100 Zigarettenkippen am VNTR locus D1S80; sämtliche Zigarettenkippen von den Tatorten). Die Resultate lassen den Schluß zu, daß die DNA-Typisierung mittels PCR eine mögliche Methode zur Analyse von Speichelspuren an Zigarettenkippen darstellt.

Schlüsselwörter: Zigaretten – Speichel – DNS – Polymerase chain reaction (PCR) – HLA-DQ alpha – D1S80 (pMCT118) – AMP-FLP – Identifikation

Introduction

Since conventional serological means of typing saliva are limited [1], it would be desirable to develop genetic marker typing procedures for characterization of traces of saliva on cigarette butts. DNA can be isolated from epithelial cells present in saliva (buccal swabs), therefore it might be possible to extract DNA from cigarette butts after mouth contact. Moreover, the polymerase chain reaction (PCR) offers the potential to amplify subanalytical quantities of DNA to analytical levels. Thus, genetic markers amenable to PCR [e.g. the HLA-DQ alpha locus as well as variable number of tandem repeat (VNTR) loci] may provide approaches for typing saliva on cigarette butts.

This paper describes a method for extraction of DNA from cigarette butts, which subsequently can be amplified by PCR at the HLA-DQ alpha [2] and D1S80 loci [3, 4]. The amplified DNA was subjected to reverse dot-blot analysis or high resolution polyacrylamide gel electrophoresis, respectively.

Materials and methods

Biological samples

Ten individuals each smoked 20 cigarettes, providing a total of 200 cigarette butts for this study. Each cigarette butt was removed from the ash tray (which was a single, generally used ashtray),

Offprint requests to: M. N. Hochmeister, Department of Forensic Medicine, Bühlstrasse 20, CH-3012 Bern, Switzerland

using forceps and maintained in a paper bag at ambient temperature and humidity for 4 weeks prior to analysis.

From those 10 individuals blood was drawn by fingerprick, placed on cotton cloth, air dried, and stored in a paper bag at ambient temperature and humidity for 4 weeks prior to analysis.

Three cigarette butts (from adjudicated cases) were collected from 2 crime scenes: one from a sidewalk and two from an ash tray. They were maintained at ambient temperature and humidity in a paper bag prior to analysis (the first case was stored for 7 months and the second cases was stored for 3 months). From the 2 individuals, who had been associated with the cigarette butts, blood was drawn by fingerprick, placed on cotton cloth, air dried and stored in a paper bag at ambient temperature and humidity for 2 weeks prior to analysis.

DNA extraction

DNA extraction from cigarette butts. Cigarette butts were handled with forceps at all times. From the end of the cigarette butt that would have been in contact with the mouth 3 cross sectional slices, each 3–5 mm wide, were made using a sterile scalpel blade. The outer paper covering from the 3 sections was removed using sterile forceps and was placed in a single 1.5 ml Saarstedt tube with screw cap. One ml of Chelex solution (Chelex 100, Biorad Richmond, CA; 5% w/v in sterile water) was added and the tube was vortexed vigorously for 30 s. After an incubation period of 30 min at 56°C the tube was vortexed again vigorously for 30 s and then boiled for 8 min. After boiling the tube was vortexed for 30 s. The outside of the tube was cleaned with ethanol and a sterile needle was used to punch a hole in the bottom of the tube. The Chelex solution was transferred to a new tube by the piggyback method [5] via centrifugation at 14000 rpm for 2 min. After removing the upper tube, the tube containing the extractant was centrifuged at 14000 rpm for 3 min to pellet the remaining Chelex. The DNA-containing supernatant was transferred to a Centricon™100 microconcentrator tube. Alternatively, after the boiling step, the tube could be vortexed for 30 s and the Chelex pelleted by centrifugation at 14000 rpm for 3 min. Then the supernatant could be transferred to a Centricon™100 microconcentrator tube. Using this approach, special care should be taken not to transfer traces of Chelex.

The Centricon™100 microconcentrator tubes were subjected to centrifugation at 4000 g and the retentate was washed with 2 ml of TE buffer (0.01 *M Tris*, 0.001 *M* $Na_2EDTA{\cdot}2H_2O$, pH 7.5) by subjecting it to a second centrifugation step. The retentate (final volume approximately 20–30 µl) was stored at 4°C until slot-blot quantitation of the recovered DNA.

DNA extraction from blood. Bloodstains were extracted according to previously described protocols [6, 7]. Briefly, a 3 × 3 mm cutting of the stain was placed in a 1.5 ml Saarstedt tube with a screw cap. One ml of sterile water was added and the stain was soaked at room temperature for 30 min. The tube was centrifuged for 3 min at 14000 rpm and all but 40 µl of the supernatant was removed and discarded. A Chelex 100 suspension (5% w/v) was added to the tube to a final volume of approximately 200 µl. Tubes were vortexed vigorously for 30 s, incubated for 30 min at 56°C, vortexed again for 30 s, boiled for 8 min, and vortexed again for 30 s. The Chelex was pelleted by centrifugation at 14000 rpm for 3 min. The supernatant was used for subsequent analysis.

Quantification of human DNA

The quantity of human DNA extracted from each sample was determined using the method of Waye et al. [8]. Briefly, 1 or 2 µl of the purified DNA extract (5–10% of the sample volume) was immobilized on a nylon membrane (Zeta Probe®, Bio-Rad Laboratories, Richmond, CA) using a slot-blot vacuum apparatus (Bio-Rad Laboratories, Richmond, CA). Subsequently, the membrane was subjected to hybridization with p17H8 (D17Z1; Oncor, Gaithersburg, MD).

Analytical and typing methods

Amplification and typing of the HLA-DQ alpha locus. 2 ng of human DNA was amplified by PCR using the AmpliType™ HLA-DQ alpha Forensic DNA Amplification and Typing Kit (Cetus Corporation, Emeryville, CA) according to the recommended protocol. Positive and negative controls were used. Typing of the HLA-DQ alpha locus was performed using a reverse dot-blot format and allele specific oligonucleotide probes [2] following the recommended protocol.

Amplification and typing of the VNTR locus D1S80 (pMCT118) – amplified fragment length polymorphism (or AMP-FLP). Amplification of the VNTR locus D1S80 was achieved using a method slightly modified from the described previously by Budowle et al. [3] and Kasai et al. [4]. Briefly, 2 ng of human DNA derived from the cigarette butts and 2–20 ng of DNA derived from bloodstains was amplified in a 25 µl PCR mixture in 0.2-ml thin walled MicroAmp™ tubes, using the Perkin-Elmer Cetus GeneAmp PCR System 9600 [9]. The thermal cycling conditions were: 95°C 20 s/65°C 20 s/70°C 4 min/30 cycles. Control samples contained 2 ng human DNA or no DNA. Electrophoretic analysis of the PCR products was performed using a high resolution horizontal rehydratable polyacrylamide gel or a vertical polyacrylamide gel electrophoresis technique, as described by Budowle et al. [3] and Hochmeister et al. [10], respectively. The gels were stained with either silver or ethidium bromide.

Table 1. Extraction, amplification and typing results from DNA derived from cigarette butts from a repetitive study

No.	Cig. butts	DNA Yield Range	DNA average per cigarette	HLA-DQ alpha pos. without BSA	HLA-DQ alpha pos. with BSA	D1S80 pos. without BSA	D1S80 pos. with BSA
1	20	5 –160 ng	29 ng	5 out of 10	10 out of 10	1 out of 10	10 out of 10
2	20	20 –160 ng	110 ng	10 out of 10	10 out of 10	8 out of 10	10 out of 10
3	20	5 – 40 ng	21 ng	10 out of 10	10 out of 10	9 out of 10	10 out of 10
4	20	20 –160 ng	76 ng	10 out of 10	10 out of 10	9 out of 10	10 out of 10
5	20	5 – 40 ng	20 ng	10 out of 10	10 out of 10	10 out of 10	10 out of 10
6	20	5 – 40 ng	17 ng	10 out of 10	10 out of 10	10 out of 10	10 out of 10
7	20	5 – 80 ng	32 ng	10 out of 10	10 out of 10	9 out of 10	10 out of 10
8	20	2.5– 20 ng	9 ng	10 out of 10	10 out of 10	8 out of 10	10 out of 10
9	20	2.5– 20 ng	10 ng	10 out of 10	10 out of 10	10 out of 10	10 out of 10
10	20	<2[a] – 80 ng	26 ng	9 out of 10[a]	9 out of 10[a]	9 out of 10[a]	9 out of 10[a]
Total:	200	<2.0–160 ng	35 ng	94%	99%	83%	99%

[a] Insufficient quantity of DNA (< 2.0 ng) in one sample

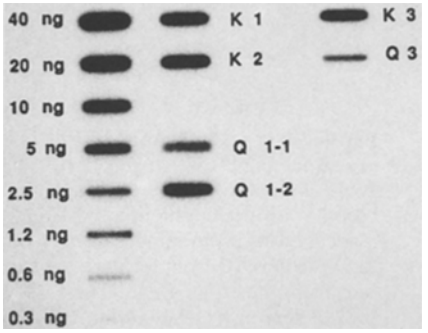

Fig. 1. Slot-blot of DNA extracted from 3 cigarette butts (Q 1-1; Q 1-2; Q 3) found at crime scenes. *First case*: K1: DNA extracted from a bloodstain produced from suspect's whole blood (5 µl out of 200 µl were used for slot-blot analysis). K2: DNA extracted from a bloodstain produced from murdered victim's whole blood (5 µl out of 200 µl). Q 1-1 and Q1-2: DNA extracted from 2 cigarette butts (3 months old; 2 µl out of 20 µl). *Second case*: K3: DNA extracted from a bloodstain produced from suspect's whole blood (5 µl out of 200 µl were used for slot-blot analysis). Q 3: DNA extracted from one cigarette butt (7 months old; 0.5 µl out of 24 µl). The serially diluted human genomic DNA standard is from K562 cell line (Promega)

Results and discussion

Human DNA was extracted from all 200 samples from the repetitive cigarette butt study, as well as from the 3 cigarette butts found at crime scenes. The amount of human DNA, which was determined by slot-blot analysis, ranged from < 2.0–160 ng per cigarette butt. The average amount of DNA recovered was 35 ng (Table 1). The 3-month-old and 7-month-old cigarette butts from crime scenes yielded 8 ng, 50 ng and 100 ng of human DNA, respectively (Fig. 1; Table 2). It has been our experience, though, that if cigarette butts are extracted within 24 h or a few days of being smoked, as much as 800 ng of human DNA can be recovered (data not shown).

Since it can be anticipated that the majority of cigarette butt samples, particularly those derived from crime scenes, will not provide a sufficient quality and/or quantity of DNA for restriction fragment length polymorphism analysis, DNA typing was attempted using PCR – based methods. The DNA from 200 cigarette butts was subjected to PCR and subsequently analyzed for HLA-

Table 2. Extraction, amplification and typing results from DNA derived from a crime scene samples study

Case no.	# cigarette butts	DNA yield	HLA-DQ alpha pos.[a]	D1S80 pos.[b]
1	1 (analyzed 7 months after recovery)	8 ng	all samples typeable	all samples typeable
2	2 (analyzed 3 months after recovery)	50 and 100 ng	all samples typeable	all samples typeable

[a] For HLA-DQ alpha all samples were initially amplified in the presence of BSA (160 µg/ml)
[b] For D1S80 the three samples were amplified in the absence of BSA

Fig. 2. Typing of HLA-DQ alpha locus after PCR using reverse dot-blot strips. DNA extracted from the butts of 10 cigarettes smoked by one individual (Cig. Nos. 1–10) yielded results identical to the control blood sample (HLA-DQ alpha Type 1.2,3)

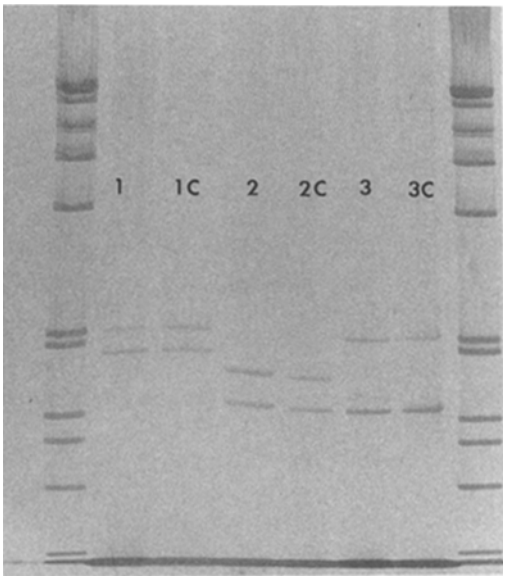

Fig. 3. Silver stained AMP-FLP gel displaying D1S80 profiles comparing DNA extracted from cigarette butts and whole blood from 3 known donors. Samples 1, 2, and 3 are from different individuals and are D1S80 profiles derived from DNA extracted from bloodstains. Samples 1 C, 2 C, and 3 C are D1S80 profiles derived from DNA extracted from cigarette butts. The amplified DNA was subjected to high resolution horizontal polyacrylamide gel electrophoresis. The size ladder is the 1 kb ladder from BRL (Gaithersburg, MD)

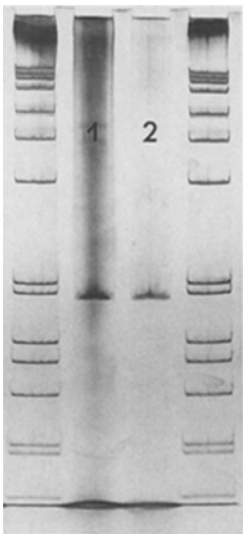

Fig. 4. Silver-stained AMP-FLP gel displaying D1S80 profiles. *Lane 1*: DNA derived from whole blood of a suspect potentially associated with the sample. *Lane 2*: DNA derived from a cigarette butt from the crime scene. The profile demonstrates that the suspect is a potential contributor of the sample. The D1S80 type of both samples is 9-9. The amplified DNA was subjected to high resolution vertical polyacrylamide electrophoresis. Lane 1 and 4: 1 kb ladder from BRL (Gaithersburg, MD)

DQ alpha and D1S80. Of the 20 cigarettes smoked by each individual the DNA extracted from 10 cigarettes was subjected to HLA-DQ alpha typing and the other 10 samples were typed for D1S80. HLA-DQ alpha typing was initially successful on 94 out of 100 samples. However, amplification was made possible on five of the six remaining DNA samples by addition of bovine serum albumin (BSA) to the PCR (at a final concentration 160 µg/ml) [10, 11]. Thus, 99 out of 100 samples were typeable for HLA-DQ alpha and the one sample which failed to type had less than 2.0 ng of human DNA which is insufficient for analysis. In one sample an extra allele due to contamination was identified. The contaminating allele was self-evident and was less intense than the correct and major-contributing profile. The positive and negative controls showed no evidence of contamination. Perhaps the contamination was due to someone else other than the control individual initially lighting the cigarette and then handing it over to the control individual. The remaining 98 samples yielded the same typing results as the bloodstain controls (Fig. 2).

AMP-FLP analysis of D1S80 was initially successful on 83 of 100 samples. All interpretable results were consistent with the bloodstain controls. BSA was not initially added to the PCR of D1S80 for samples which were difficult to amplify, because the presence of BSA would lead to excessive lane background due to silver staining after electrophoresis. However, after addition of BSA to the PCR (at a final concentration of 160 µg/ml) all but one sample amplified (i.e., 99 samples). These samples with BSA were run on vertical polyacrylamide gels [10] and typing was possible after staining with ethidium bromide. The only differences between analysis on ultrathinlayer silver stained gels and ethidium bromide stained gels were that silver stained gels provided permanent records and less sample volumes of the PCR were required for analysis of amplified products (4–7 µl vs 25 µl).

Analysis of the cigarette butts from the adjudicated cases was for research purposes only and solely to indicate whether or not the procedure described in this paper is feasable on evidentiary material. Amplification of the HLA-DQ alpha locus from the DNA from 3 cigarette butts recovered from crime scenes was possible only after the addition of BSA to the PCR. It has been suggested by Rogan and Salvo [12], that BSA may bind a soluble inhibitory factor for PCR that copurifies with DNA. In the first case (7-month-old cigarette butt) the HLA-DQ alpha type of the cigarette butt was 1.1, 1.2. and the blood sample of the suspect was 2,2. Therefore, the suspect was excluded as a potential contributor of the sample. The D1S80 profile also demonstrated an exclusion. This exclusion is consistent with earlier evidence in the case by which the suspect had been exonerated.

In the second case (3-month-old cigarette butts) the HLA-DQ alpha type derived from the cigarette butts recovered from the crime scene and the suspect's blood were both 1.3,4, whereas the murdered victim's blood type was 1.1,4. The D1S80 profiles from the cigarette butt and suspect's blood (9-9) also matched (Fig. 4). Thus, the suspect was included as a potential contributor of the sample. This inclusion is consistent with an earlier confession by the suspect that he had smoked the cigarette found at the crime scene.

It is important to stress, that an inclusion (or for that matter an exclusion) via DNA typing provides no information whether or not a crime has been committed; it only suggests whether or not the evidentiary material potentially could have originated from the suspect. Since some population data are available for HLA-DQ alpha and D1S80, statistical weight (i.e. the portion of the

population that are potential contributors) can be provided for the second case. The frequency of a 1.3,4 HLA-DQ alpha type and a 9-9 D1S80 type is 0.065 and 2.56×10^{-4}, respectively [data derived from Budowle et al. [3] and Budowle et al. (in preparation)]. The 2 types combined suggest that approximately only one out of every 60000 individuals in the Caucasian population are potential contributors.

For some samples in this study amplification proved to be difficult and the inability to amplify DNA derived from some cigarette butts was overcome by the addition of BSA to the PCR. Since the addition of BSA to the PCR for those few samples which did not initially amplify, was necessary, it may be prudent to consider the initial additon of BSA to samples of limited quantity where only one amplification is possible. Furthermore, amplification failed in some bloodstain control samples where no water presoak was carried out prior to Chelex extraction (data not shown). Therefore, a water presoak step is recommended for bloodstains to yield DNA that is suitable for PCR.

Finally, slot blot analysis should be considered an important part of the process when attempting PCR on the kind of samples described in this paper. It allows the determination of the total quantity of extracted human DNA; and, therefore, permits efficient aliquoting of the DNA to amplify several PCR-based genetic marker systems. Additionally, a prior knowledge of the quantity of DNA used in the PCR is useful for AMP-FLP analysis. For example, when amplifying 100 ng of human DNA for D1S80 a maximum of 25 cycles of PCR should be employed; however, if 2 ng of human DNA is subjected to PCR, 30–35 cycles of PCR are appropriate. Also, a positive result on a slot-blot indicates that DNA of human (or at least higher primate) origin is present in the sample.

In conclusion, PCR-based analysis of DNA extracted from saliva on cigarette butts offers the potential to genetically characterize material where it was not previously possible with conventional serological marker typing. The results indicate that sufficient quantities of DNA for PCR of HLA-DQ alpha and D1S80 (and for that matter additional genetic markers) could be extracted from cigarette butts and the vast majority of samples are amenable to typing. Therefore, this paper provides additional support for the reliable use of PCR-based typing procedures for the analysis of forensic biological materials. Currently, we are investigating additional genetic marker systems suitable for analysis as well as attempting to develop more effective conditions to improve the efficiency of PCR.

Names of commerical manufacturers are provided for identification only and inclusion does not imply endorsement by the Federal Bureau of Investigation.

References

1. Biology Methods Manual (1978) Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE 17 LP, England, Section 3–12
2. Saiki R, Walsh PS, Levenson CH, Erlich HA (1989) Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc Natl Acad Sci USA 86:6230–6234
3. Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC (1991) Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. Am J Hum Genet 48:137–144
4. Kasai K, Nakamura Y, White R (1990) Amplification of a variable number of tandem repeat (VNTR) locus (pMCT188) by the polymerase chain reaction (PCR) and its application to forensic science. J Forensic Sci 35:1196–1200
5. Kimes DR, Tahir MA (1985) An extraction procedure for seminal/vaginal stains to eliminate streaking in the electrophoresis of phosphoglucomutase. Crime Laboratory Digest 12: 32–33
6. Singer-Sam J, Tanguary RL, Riggs AD (1990) Use of chelex to improve the PCR signal from a small number of cells. Amplifications 3:11
7. Jung JM, Comey CT, Baer DB, Budowle B (1991) Extraction study for obtaining DNA from bloodstains for PCR amplification and typing of the HLA-DQ alpha gene. Int J Leg Med 104:145–148
8. Waye JS, Presley LA, Budowle B, Shuttler GG, Fourney RM (1989) A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. Bio Techniques 7:852–855
9. Haff L, Atwood JG, DiCesare J, Katz E, Picozza E, Williams JF, Woudenberg T (1991) A high-performance system for automation of the polymerase chain reaction. Bio Techniques 10:102
10. Hochmeister MN, Budowle B, Borer UV, Eggmann UT, Comey CT, Dirnhofer R (1991) Typing of DNA extracted from compact bone from human remains. J Forensic Sci (in press)
11. Hagelberg E, Sykes B, Hedges R (1989) Ancient bone DNA amplified. Nature 342:485
12. Rogan PK, Salvo J (1990) Study of nucleic acids isolated from ancient remains. Yearbook of Physical Anthropology, vol 33, pp 195–214