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Diagnosis of HIV infection from bloodstains by PCR. A further marker for identification

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Abstract The forensic usefulness of the detection of HIV infection in bloodstains is linked to the increasing spread of HIV infection and the consequent rise in the number of forensic cases involving HIV-positive subjects. This study was designed to detect HIV infection in bloodstains of various ages obtained from HIV-positive patients treated with zidovudine (3′-azido-3′-deoxythymidine, AZT) using PCR methods. Of the 3 kinds of extraction and amplification methods checked, only a nested PCR method for the amplification of a sequence located in the HIV Reverse Transcriptase (RT) region was successful. This method, involving 2 amplification steps (1: fragment of 768 bp; 2: codons 41-67-70-215-219), encompasses the mutations commonly observed during AZT therapy and overcomes the limitations inherent in serological testing. The discriminatory power of the method can detect specific mutation patterns in the RT gene linked to drug resistance and compares the specific pattern of the bloodstain with fresh blood or other specimens from the subject in question.

Key words Bloodstains · Individual identification · PCR · HIV

Introduction

Individual identification from bloodstains can use both genetic and non-genetic markers, the latter being understood as distinctive properties of the organism which constitute individual identification, e.g. biochemicals and antibody profiling, persistent disease agents and others (Merli et al. 1980; Sensabaugh 1982). The forensic application of such tests has progressively decreased as a consequence of the rapid development in the 1970s and 1980s of sensitive, reliable techniques for detecting the phenotypical characters of blood cell components and serum proteins. Also, the mid-1980s saw the rise of techniques for examining DNA polymorphisms (e.g., RFLP, VNTR, STR, HLA systems) (Jeffreys et al. 1985; Nakamura et al. 1987; von Beroldingen et al. 1989; Kirby 1990; Berghaus et al. 1990; Robertson et al. 1990; Rand et al. 1992; Edwards et al. 1992; Kimpton et al. 1992; Wiegand et al. 1993).

Of the non-genetic characters, HIV infection takes on particular importance, due to its prevalence in many developing and industrialized countries and the increasing number of forensic cases in which the biological material to be identified is composed of traces left by HIV carriers.

Some authors (Stiebler et al. 1989; Pappalardo et al. 1991) thus proposed tests allowing the diagnosis of HIV infection by identification of specific antibodies in bloodstains. However, methods based on the search for specific antibodies do not allow identification of 1) individuals with delayed serological response to the virus, 2) infected patients with indeterminate serological results, 3) patients with dual retroviral infections, nor do they discriminate subgroups of infants born from seropositive mothers (antibodies of newborn or maternal origin) (Coutlee et al. 1991).

These limitations, involving testing on both fresh blood and bloodstains, have been overcome on fresh blood by the PCR in vitro amplification technique, which allows the detection of viral gene sequences integrated in the genome of peripheral blood lymphocytes.

The aim of the present work was to report the results of a study on the applicability of this technique to bloodstains by testing 3 amplification methods derived from those used on fresh blood (Loche and Mach 1988; Ou et al. 1988; Larder et al. 1991), each tested with the DNA yielded by 3 different extraction methods.

The possible fields of forensic application of this method, its potentials and limitations, together with common haemogenetic markers for individual identification in a forensic framework, are all considered.

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Materials and methods

Whole blood samples from 5 HIV-positive subjects in out-patient pharmacological treatment with zidovudine (3′-azido-3′-deoxythymidine, AZT) at doses of 3.5 mg/kg × 6/day were used to prepare bloodstains. Aliquots of 200 µL of whole blood were deposited on 3 different substrates: absorbent paper, cotton and plastic film.

The samples were kept at room temperature in non-sterile conditions for periods of either 6 months or 2 years, and then examined with 3 different extraction methods. Each extract was then processed according to 3 different amplification protocols. Electrophoretic separation was carried out as reported below.

Extraction

Method 1 Phenol-isoamyl alcohol method based on the procedure developed by Budowle and Baechtel (1990).

Method 2 Based on the HIV-1 Amplicor commercial kit (Art. 0743925 US: 83003, Roche, Switzerland) and manufacturer's instructions: 1.0 mL of washing solution was placed in a 2-mL Sarstedt test-tube containing a 3 × 3 mm sample. The tube was hermetically sealed, inverted a few times, and incubated at room temperature for 5 min. These 2 operations were then repeated. The sample was centrifuged for 3 min at maximum speed. The supernatant was aspirated taking care not to remove the pellet. The extraction buffer was brought to room temperature and vortexed. Extraction buffer (200 µl) was added to the pellet, revortexed and incubated at 60° C for 30 min in a dry thermostatted cell. The extract was finally incubated at 100° C for 30 min.

Method 3 Chelex method as follows: 500–1000 µl 1 × TE was added to a 3 × 3 mm sample in 1.5 mL screw-capped Eppendorf tube. The mixture was kept at room temperature for 12 h. The solution was then centrifuged at 13,000 rpm for 3 min and the supernatant was discarded. A 200 µL aliquot of 5% Chelex (Art. 142-2832, Bio-Rad Richmond, Va.) was added to the pellet. Samples were incubated at 56° C for 30 min and after vortexing for 20 s, the solution was boiled at 100° C for 8 min. After vortexing for 10 s, the solution was spun for 3 min at 13,000 rpm (Walsh et al. 1991).

Amplification

Amplifications were carried out in a DNA thermal cycler (Perkin Elmer Cetus, USA) under the following conditions:

Method 1

30 cycles of denaturation at 92° C for 1 min, annealing at 40° C for 1 min, and extension at 70° C for 3 mins. DNA samples (0.1 µg or 10 µL of Chelex extract) and 1 µg of each primer were aliquoted into a 0.5-mL Eppendorf tube containing the reaction mix up to a final volume of 100 µL (Loche and Mach 1988).

Two units of Taq polymerase were added after the first denaturing step.

Primer sequences 5′-GAAGGAGAGAGATGGGTGCG-3′ and 5′-GGGATGGTTGTAGCTGTCCC-3′ corresponded to the flanking region of a 213 bp target sequence of a segment of the HIV-1 gag gene.

Method 2

30 cycles of denaturation at 95° C for 1 min, annealing and extension at 60° C for 1 min. Amplification was performed in a 100 µL reaction mix containing 200 µM each of 4 dNTP's 1 × PCR Buffer II (Perkin Elmer Cetus, USA), 2.5 mmol $MgCl_2$, 0.1 µg of DNA sample (or 10 µL of Chelex extract) and 0.1 µg of each primer. Two units of Taq polymerase were added after the first denaturing step (Ou et al. 1988).

Primer sequences 5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′ (SK 38) and 5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′ (SK 39) corresponded to the flanking region of a 115 bp target sequence of a segment of the HIV-1 gag gene.

Method 3

This involved a nested PCR approach. In the first step (a), a fragment of 768 bp from within the Reverse Transcriptase (RT) region (total length 1.7 kb) was amplified. In the second step (b), the product of the first amplification was used with different primer pairs that recognize codons 41, 67, 70, 215 and 219 where commonly observed AZT mutations have been mapped (Larder et al. 1991). The precise description of steps (a) and (b) is as follows:

(a)-First step (common).

30 cycles of denaturation at 94° C for 1 min, annealing at 45° C for 1 min, extension at 72° C for 2 mins. The first denaturing cycle lasted 8 mins, the last extension period was 10 mins.

The reaction mix (100 µL) was made of 0.1 µg of DNA sample (or 10 µL Chelex extract), 0.2 mmol dATP, dGTP, dCTP and dTTP, 0.25 µg of each primer and 2.5 U Taq DNA-polymerase, in a reaction buffer containing 25 mmol KCl, 50 mmol Tris-HCl pH 8.3, 0.1 mg BSA and between 1.5 and 8 mmol $MgCl_2$. The solution was overlaid with 100 µL of mineral oil. The primer sequences were: 5′-TTCCCATTAGTCCTATT-3′ (Primer A) and 5′-TCATTGACAGTCCAGCT-3′ (Primer NEI).

(b)-Second step.

Two reactions, one with the wild type primer and one with the mutant primer, were performed in parallel for each codon. The reaction mix was the same as in step (a). The DNA sample consisted of 10 µL preamplified material, diluted 1 : 100. The primers (W for wild type and M for Mutant) and conditions for each amplification were essentially those described by Larder et al. (1991) and are reported below:

Codon	Primers	Cycles	Denaturation	Annealing	Extension
41	A + 5W5M	30	94° C/1 min.	50° C/1 min.	72° C/1 min.
67	A + 1W1M	30	94° C/1 min.	45° C/1 min.	72° C/2 min.
70	A + 2W2M	30	94° C/1 min.	40° C/1 min.	72° C/2 min.
215	B + 3W3M	30	94° C/1 min.	45° C/1 min.	72° C/2 min.
219	B + 4W4M	30	94° C/1 min.	40° C/1 min.	72° C/2 min.

W = wild type; M = mutant.

The first denaturing cycle lasted 8 min, the last extension period was 10 min. The primer sequences (Larder et al. 1989) were:

Primer B: 5′-GGATGGAAAGGATCACC-3′
Primer 1W: 5′-TTTTCTCCATTTAGTACTGAC-3′
Primer 1M: 5′-TTTTCTCCATCTAGTACTGAT-3′
Primer 2W: 5′-AATCTACTAATTTTCTCCAAT-3′
Primer 2M: 5′-AATCTACTAATTTTCTCCAAC-3′
Primer 3W: 5′-ATGTTTTTTGTCTGGTGTGGT-3′
Primer 3M: 5′-ATGTTTTTTGTCTGGTGTGAA-3′
Primer 4W: 5′-AGGTTCTTTCTGATGTTTTAT-3′
Primer 4M: 5′-AGGTTCTTTCTGATGTTTTAG-3′
Primer 5W: 5′-AATTTTCCCTTCCTTTTCCTT-3′
Primer 5M: 5′-AATTTTCCCTTCCTTTTCCTA-3′

Gel analysis

Aliquots of 10 µL of the amplified product with 2 µL loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in sterile water) were loaded onto a 3% agarose gel (Nusieve 3 : 1) in 1 × TBE. After electrophoretic separation, the gels were stained with ethidium bromide. Visualization was carried out under UV light.

Table 1 Lengths of amplified fragments

Codon 41	243 bp
Codon 67	221 bp
Codon 70	227 bp
Codon 215	210 bp
Codon 219	222 bp

Results

Neither amplification methods 1 and 2 (both aiming at amplifying the gag gene) were able to provide visible bands of specific amplified fragments with some of the substrates used and with the various types of extraction. On the contrary, the nested PCR method did amplify the target sequences from all types of bloodstain, when a Chelex extraction procedure or the HIV-1 Amplicor extraction mixture were used. Well-defined amplification bands were obtained from bloodstains prepared as long as two years before testing.

Table 1 shows the lengths of the amplified fragments, identical for both wild type and mutant sequence.

The amplification profiles (Figs. 1 and 2) were consistent with the patterns described below.

– Visible amplification bands obtained with both wild type and mutant primers, with identical quantitative ratios (lanes 1 and 2 in both figures). This pattern was suggestive of the simultaneous presence of 2 viral populations in the same patient.

– Visible amplification bands obtained with both wild type and mutant primers at different intensities (Fig. 1: lanes 3 and 4, 11 and 12). This pattern was suggestive of the simultaneous presence of 2 viral populations in the same patient, although in different quantitative ratios. The same quantitative ratio was reproduced starting from different types of bloodstains from the same subject;

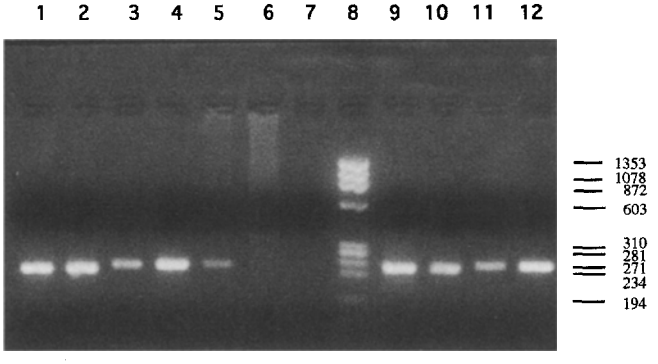

Fig. 1 Selective amplification products of DNA fragments from reverse transcriptase (RT) region of HIV gene. Extraction with Chelex. Lanes 1–6 paper; lanes 9–12: cotton. Lane 1: codon 215 wild type (WT); lane 2: codon 215 mutant (M); lane 3: codon 219 WT; lane 4: codon 219 M; lane 5: codon 67 WT; lane 6: codon 67 M; lane 7: H_2O; lane 8: DNA molecular weight-marker IX (Boehringer); lane 9: codon 215 WT; lane 10: codon 215 M; lane 11: codon 219 WT; lane 12: codon 219 M. Marker size (bp) is shown on right

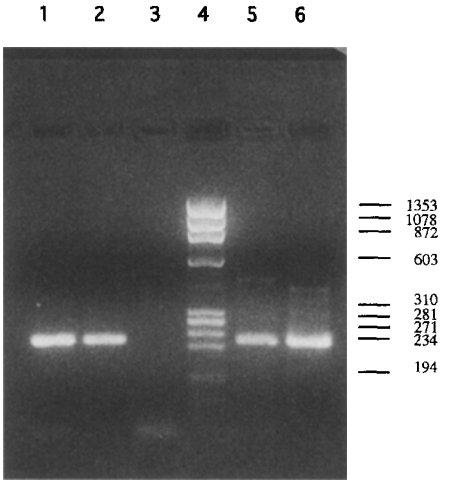

Fig. 2 Selective amplification products of DNA fragments from reverse transcriptase (RT) region of HIV gene. Extraction with Amplicor kit (Roche, Basel, Switzerland). Lanes 1–2: cotton; lanes 5–6: nylon. Lane 1: codon 67 wild type (WT); lane 2: codon 67 mutant (M); lane 3 H_2O; lane 4: DNA molecular weight-marker IX (Boehringer); lane 5: codon 215 WT; lane 6: codon 215 M. Marker size (bp) is shown on right

– Visible amplification band obtained with only one primer (Fig. 1: lanes 5 and 6) indicating that only one viral population was present in the patient at that moment.

Discussion

The method based on a single amplification step for a gag sequence target (Ou et al. 1988; Loche and Mach 1988) did not provide reliable results with either phenolchloroform or Chelex sample extraction.

The nested PCR method (Larder et al. 1989) which adopts wild type and mutant primers in parallel for the same codon of the RT gene, allowed the detection of HIV infection from extremely small bloodstains. This implies that a method reliable for fresh blood cannot guarantee the same efficiency on bloodstains.

Studies on fresh blood carried out by the nested PCR method (Larder et al. 1991; Chow et al. 1993) have shown that the virus infecting peripheral blood lymphocytes (PBL) during AZT therapy accumulates specific predictable mutations in the RT coding sequence associated with drug resistance.

Mutations arising in the RT region cause specific aminoacid substitutions at the following residues: Met41 → Leu, Asp67 → Asn, Lys70 → Arg, Thr215 → Phe or Tyr, Lys219 → Gln (Larder et al. 1991; Kellam et al. 1992). It has been observed that the mutation at codon 70 commonly occurs first during AZT therapy in asymptomatic patients. This mutation is observed only transitorily and the viral population is later replaced by a stable virus population mutant at codon 215. It has also been shown how prolonged treatment is necessary before the partially resistant population becomes highly resistant by means of

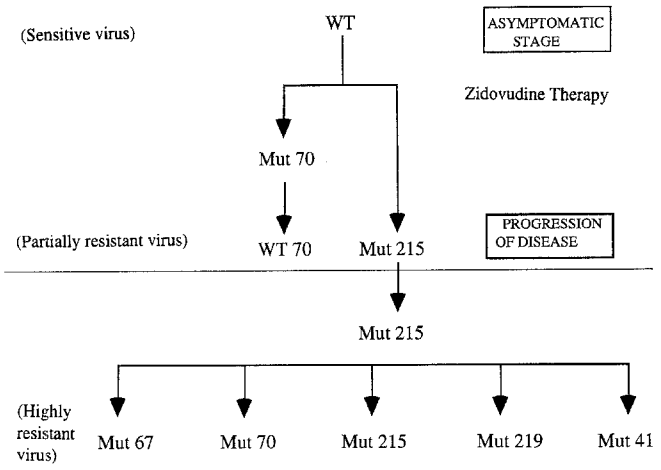

Fig. 3 Proposed model for sequential events in zidovudine treatment of asymptomatic individuals (from Boucher et al. with modifications)

the acquisition of additional specific mutations – a phase clinically associated with progression of the disease. The chronological order of appearance of mutations is shown in Fig. 3 (Boucher et al. 1992). The fifth mutation (codon 41) has been detected only after the appearance of the codon 215 change in the RT coding sequence (Kellam et al. 1992).

The results of our studies show that amplification by the nested PCR method is possible when extraction is carried out by Chelex, which prevents DNA degradation by chelating metal ions that function as catalysts in the breakdown of DNA at high temperatures in low ionic strength solutions. However, the HIV-1 Amplicor extraction mixture currently used on whole blood is a good alternative to Chelex.

The method allows the diagnosis of HIV infection in bloodstains of various kinds and ages, overcomes the limitations of serological testing and can answer the question of the HIV infectivity of the subject at the moment of leaving the stain.

Evaluation of the results in any forensic case must consider that HIV infection is a stable marker which can, however be acquired in the period between the deposition of the bloodstain and its examination.

The method supplies further information through the recognition of the specific mutation patterns which occur during AZT therapy.

Patterns must be evaluated carefully, bearing in mind their number, type and age of bloodstain (if known). In particular the two main following situations can appear:

A) The bloodstain found on a suspect comes from a dead person: the comparison of mutation patterns both of the blood of the victim and of the stain gives maximum information, with exclusion in case of mismatch and a high degree of individual identification in case of perfect match.

B) The bloodstain found on a crime scene comes from a living person: because the mutation pattern is subject to chronological variations, the length of time elapsed is a determinant factor. In any case a stain positive for HIV infection with any mutation pattern can exclude: 1) HIV-negative subjects, 2) HIV-positive subjects with mutation patterns chronologically antecedent to those of the stain. If the same mutation pattern is found on the suspect and the stain is recent, there is a high degree of probability of identification. In all other cases, e.g., stains of unknown age, or different but chronologically compatible mutation patterns, conclusions must be drawn with care.

Considering its potentials, the method constitutes a further useful marker of identification for the forensic laboratory. In cases of HIV-positive subjects it may represent a screening method. Its usefulness will increase with better knowledge of the mutation rate of the RT region and with improved detection technologies such as quantitative PCR of mutant and wild types or RT region sequencing.

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