Int J Legal Med (2001) 114 : 295–297

SHORT COMMUNICATION

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Variations in primer sequences are the origin of allele drop-out at loci D13S317 and CD4

Received: 2 June 2000 / Accepted: 19 September 2000

Abstract STRs have become almost the exclusive tool of genetic scientists in forensic typing work. Consequently, large numbers of samples are genotyped and the detection of rare abnormalities is to be expected. We found rare losses of alleles, also known as drop-out, at the two STR loci D13S317 and CD4. Drop-out at D13S317 was accidentally found in typing of suspects in a murder case and three other examples of drop-out were found at locus CD4 during paternity testing. The lost alleles reappeared when alternative PCR primer pairs were used. Sequences of lost alleles were characterised at the molecular level after cloning. Variations were found in the primer sequences and these are believed to prevent amplification or to reduce amplification yield and to be the origin of the allele drop-out.

Keywords Short tandem repeat · D13S317 · CD4 · Allele drop-out · Primer sequence variation

Introduction

DNA typing with short tandem repeat systems (STRs) has become the major methodology to obtain individual genetic profiles and is widely used in criminal and paternity investigations. PCR amplification of these polymorphic repeat loci is used routinely to establish large numbers of genotypes. Amplification depends on hybridisation with locus-specific primer sequences enclosing the repeat. Considering the polymorphic nature of the human genome, variations could be expected to occur in primer sequences. These polymorphisms could affect efficient primer/template interaction and change the product yield of the PCR reaction.

D13S317 is part of commercial DNA profiling kits and is one of the 13 core STRs typed in the CODIS system allowing interlaboratory comparison of genotypes.

Allele drop-out causes a heterozygote to be falsely recorded as homozygous, so that when searching data banks, the result may be interpreted as non-concordant. Allele drop-out in a paternity test, as found here for STR locus CD4, could be incorrectly interpreted as an exclusion. With CD4, the product yield of the PCR reaction varied and seemed to depend on amplification conditions, but with D13S317 loss of an allele was constant. Since both observations could lead to misinterpretation, we decided to investigate the underlying mechanisms of these allele drop-outs. Variation of product yield for the CD4 locus has been observed and studied before (Watanabe et al. 1998) and was associated with a polymorphism in the forward primer sequence.

Materials

The following STR profiling kits were used: AmpF*l*STR Profiler (Perkin Elmer Biosystems, Foster City, Calif.) and Gamma STR Multiplex (Promega, Madison, Wis.). Synthesised primer-pairs for amplification and for sequencing are indicated in Fig. 1. PCR products from homozygotes were sequenced directly. PCR products from heterozygotes were cloned in pGEM-T easy (Promega) and clones were selected at random and sequenced with T7 and SP6 primers. If necessary, additional clones were sequenced to obtain both alleles. Sequencing was done on an ABI PRISM 310 Genetic Analyzer using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

For CD4, the nomenclature in the literature is confusing and we tried to respect the recommendations drawn up by the ISFH DNA commission. The first repeat motif (TTTTC) on the coding strand [Genome Data Base (GDB) accession no. M86525, GenBank no. 180121] was chosen. In their studies of the CD4 locus, this nomenclature was also used by Urquhart et al. (1994), Casarino et al. (1996) and Watanabe et al. (1998). Edwards et al. (1991) defined the repeat motif as (CTTTT) and Glock et al. (1995) as (AAAAG)

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D13S317 allele 12

5'-gggttgctggacatggtatcACAGAAGTCTGGGATGTGGA---N82---(ATCT)12ATCAATC(ATCT)3TTCTGTC**TGTC**TTTTTGGGC---N36---gaccaacaattcaagctctc-3'

D13S317 allele 7 (variant)

5'-gggttgctggacatggtatcACAGAAGTCTGGGATGTGGA---N82---(ATCT)7 **ATCA**ATCAATC(ATCT)3TTCTGTCTTTTTGGGC---N36---gaccaacaattcaagctctc-3'

a

CD4 allele 6

5'-actatatgctacagatgaag---N36---TTGGAGTCGCAAGCTGAACTAGCG(TTTTC)6---N61---TTCACTGCAGCCTCAACTTCCTGG---N38---gggattataggcatgagcca-3'

CD4 allele 5 (variant)

5'-actatatgctacagatgaag---N36---TTGGAGTCGCAAGCTGAACTAG**A**G(TTTTC)5---N61---TTCACTGCAGCCTCAACTTCCTGG---N38---gggattataggcatgagcca-3'

b

Fig. 1 a, b Variations in primer sequences noted as they were found in the databases. Primer sequences normally used for the amplification are *underlined*. Upstream and downstream primers used to clone and to sequence alleles are noted in *lower-case letters*. The letter *N* followed by a number indicates the number of nucleotides between two noted sequences. **a** For D13S317, the reverse primers of the normal allele 12 contain the sequence TGTC *(bold)* while in drop-out allele 7, this sequence is deleted. The latter has an upstream 4 bp insertion of ATCA *(bold)*. **b** In the CD4 locus, the penultimate nucleotide in the forward primer sequence, C (normal allele 6), has been substituted by A in variant allele 5 *(bold)*

but this reduces the repeat number by one unit. Hammond et al. (1994) and Mertens et al. (1997) used the motif (AAAAG) but attributed incorrect allele numbers.

Results and discussion

Locus D13S317

During routine case work using the AmpF/STR Profiler kit, an off-ladder 7 allele was found in two brothers of Asiatic origin both with haplotype 7/12. Since we had never found allele 7 before in 429 unrelated persons (Egyed et al. 2000) and as it was not present in the kit ladder, we decided to characterise this rare allele by sequencing. Published primer sequences were used (GDB: G09017) as no primer sequences were available from the manufacturer of the kit. Remarkably, using this primer-pair, only allele 12 was amplified. The allele was sequenced and proved to correspond to the sequence described for locus D13S317. Using the AmpF*l*STR Profiler kit, the fragment length of allele 12 was 224 bp while our fragment had a length of 189 bp, suggesting that the primers in the kit were different from ours and localised outside our fragment. When using the Gamma STR Multiplex kit, again only allele 12 was observed. With this kit, allele 12 had a length of 185 bp, suggesting that part of the same primer sequences found in GDB were used to amplify the D13S317 locus. We reasoned that differences in primer sequences could be the origin of allele 7 drop-out. In order to check this hypothesis, we tested primers upstream of the forward and downstream of the reverse primer (Fig. 1). Again, alleles 7 and 12 were obtained. Sequencing of several clones of both alleles revealed for allele 7 a deletion of 4 nucleotides in the reverse primer sequence combined with an insertion of 4 nucleotides in the adjacent sequence (Fig. 1). This important sequence difference most probably inhibits the formation of a stable template/primer hybrid and prevents amplification of the allele.

The discovery of this drop-out allele was accidental, and our attention would not have been drawn to it if allele 7 had been present in the reference ladder of the typing kit. However, this reference allele is present in other commercial kits. We have not yet sequenced other samples of allele 7. It also remains uncertain whether other alleles could carry the same complex deletion/insertion polymorphism. In an extensive population study using the CODIS STRs (Budowle et al. 1999), the drop-out problem with D13S317 was encountered with the PowerPlex1.1 kit (Promega) which probably uses the same primers as the Gamma STR Multiplex kit (Promega). In a recent article (Budowle 2000), adaptation of primer sequences for D13S317 in the PowerPlex1.2 kit was reported.

Locus CD4

During paternity testing, we obtained two abnormal results with STR CD4. In case one, CD4 showed an exclusion while the eight other STRs of the typing system (Alford et al. 1994) did not exclude the alleged father. In case

two, no paternal alleles were found for the same STR. For both cases, however, when typing with CD4 was repeated, a faint band corresponding to allele 5 was sometimes visible for both the alleged father and the child. This suggests that in both cases, father and child share allele 5 and that paternity is no longer excluded. In the second case, the father is presumed to be homozygous. Since the sequence (GenBank U47924) and the primers used for CD4 were available (Hammond et al. 1994) we designed primers outside the first primer-pair (Fig. 1 b) to amplify and clone allele 5. Sequencing revealed a variant allele 5 sequence (Fig. 1 b) presenting a C to A nucleotide substitution in the 3′-penultimate position of the forward primer. This variant sequence has been reported before (Watanabe et al. 1998) in a study using a slightly different forward primer carrying the polymorphism in the 3′ ultimate position. Nucleotide differences in this position form the basis of allele refractory multiplication systems (ARMS) technology (Newton et al. 1989) which is frequently used to detect point mutations. However, the mispairing of variant T (template) positioned in front of C (primer) has little influence on amplification (Kwok et al. 1990) but reaction conditions affect product yield (Watanabe et al. 1998). For our primers (Hammond et al. 1994), the mismatch was in 3′ penultimate position. During experimental work on another locus aiming to enhance ARMS results by the synthetic introduction of internal primer mismatches (Besançon et al. 1998), we also found large variations in product yield depending on experimental conditions. This may lead, in the extreme case, to complete loss or drop-out of alleles. Locus CD4 is part of a triplex reaction (Hammond et al. 1994), and conditions may not be optimal for maximum product yield. To date, allele drop-out at CD4 has only been observed for allele 5, which is one of the most frequent CD4 alleles (allele 5 in Urquhart et al. 1994, Casarino et al. 1996 and Watanabe et al. 1998; indicated as allele 8 in Hammond et al. 1994, Mertens et al. 1997 and Edwards et al. 1991; indicated as allele 4 in Glock et al. 1995). CD4 is not a frequently used STR, but some laboratories still use this locus for paternity testing and should be aware of occasional drop-out of alleles. A solution could be to design alternative primers shifted either slightly or completely upstream.

In conclusion, with the routine typing of large numbers of STRs, variations in primer sequences will be found that can influence the overall product yield of PCR reactions. Some of these variations, for instance single nucleotide changes positioned at the 5′-end or near the centre of the primer sequence will not influence efficient primer extension. Other, more important changes, such as the 4-nucleotide deletion found here for D13S317 will completely abolish product formation. Some changes near the 3′-end of the primer, as found for the CD4 locus, will change product yield and could, under certain amplification conditions, cause allele drop-out. In paternity testing, the occurrence of an exclusion in homozygous father and child should be interpreted with care. Allele drop-out could be interpreted as a mutation and it is generally accepted that exclusions of paternity should never rely on a single locus. The use of different primer-pairs, as is probably the case with commercial typing kits, could lead to the compilation of different haplotype results in genetic databases. When comparing such information, search algorithms should be used that do not exclude on the basis of a single non-concordant result and, if possible, samples should be re-analysed using the same primer-pairs (Walsh et al. 1998).

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