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Problems associated with the DNA analysis of stains

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Summary. Selected stain cases are presented which highlight various problems associated with DNA investigations on stain materials, especially risks of artefacts. These cases involve stain materials (blood, semen) which were exposed to different external conditions so that the DNA was partially degraded and of variable quantity. One multi locus probe (MLP) and 4–6 single locus probes (SLP's) were used for individualisation but artefacts such as extra bands, band deficiencies or shifts only occurred with the MLP. In one case where only a few spermatozoa were present in the vaginal swab, DNA extraction was carried out without preferential lysis to avoid loss of sperm DNA. The resulting mixed band pattern could be clearly attributed to the bands from the corresponding blood samples after SLP hybridisation.

Key words: Stain cases – VNTR-Polymorphisms – Single locus probes (SLP's) – Multi locus probe (MLP)

Zusammenfassung. Anhand ausgesuchter Spurenfälle wurden verschiedene Problembereiche, insbesondere Artefaktrisiken, bei DNA-Untersuchungen an Spurenmaterial behandelt. In den vorgestellten Fällen lagen Spurenmaterialien (Blut, Sperma) in deutlich verschiedener Quantität vor, die unterschiedlichen exogenen Bedingungen ausgesetzt und dementsprechend tw. degradiert waren. Die Individualisierungsnachweise erfolgten mit 4–6 Singlelocus-Sonden (SLS's) und mit einer Multilocus-Sonde (MLS). Artefakte, wie Zusatzbanden, Defizienzen oder „shifts" ergaben sich nur mit der MLS, so daß die SLS's, auch aufgrund ihrer erhöhten Sensitivität, der MLS vorzuziehen waren. Als methodische Alternative wurde in einem Fall mit nur wenig Spermien im Vaginalsekret bei der DNA-Extraktion auf eine preferentielle Lyse verzichtet, um DNA-Verluste zu vermeiden. Das Mischbandenmuster konnte mit SLS-Hybridisierung anhand der Vergleichsblutproben von Tatverdächtigem und Opfer zweifelsfrei zugeordnet werden. In allen vorgestellten Fällen führte die SLS-Anwendung zu eindeutig auswertbaren Ergebnissen, die MLS-Anwendung teilweise zu Interpretationsschwierigkeiten.

Schlüsselwörter: Spurenfälle – VNTR-Polymorphismen – Singlelocus-Sonden (SLS's) – Multilocus-Sonde (MLS)

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Introduction

The individualisation of biological stains has been extensively studied using classical systems and much experience has been gained on the wide variety of problems which can occur (Rand et al. 1990). However, DNA investigations on stains are relatively new and experience in problem areas of DNA analysis is correspondingly low. The results can be influenced by a variety of factors such as the type of stain, storage conditions and bacterial contamination (Gill et al. 1985).

It is the aim of this paper to highlight some features which can give rise to problems in expertises on stains and should therefore be taken in consideration. If more such observations are made available this could serve as a framework to identify possible sources of error thus enhancing the evidential value.

Materials and methods

Fresh EDTA blood samples were extracted as described elsewhere (Brinkmann et al. 1991). The extraction of bloodstains from textiles such as cotton fabrics was carried out by cutting the textile into ca. 3 mm^2 pieces which were then transferred to a syringe (20 ml) containing 15 ml TE-buffer (10 m*M* Tris pH 7.5, 0.1 m*M* EDTA). The syringe was sealed with parafilm after pressing the plunger to expel the air. Extraction was carried out overnight at 4°C on an overhead shaker. Dried bloodstains on metal or synthetic materials were extracted with TE-buffer in a reaction tube and then incubated overnight as above. Aliquots of stain extract (1.5 ml) were decanted into a reaction tube (1.5 ml) and centrifuged for 5 min at 13000 rpm. The supernatant was removed, another aliquot was added and the process repeated for the whole extract. The sediment was resuspended in buffer II (10 m*M* Tris pH 8.0, 0.4 *M* NaCl, 2 m*M* EDTA) and digested with 50 µl pro-

teinase K (2 mg/ml, Pharmacia, FRG) and 12.5 µl 20 % SDS in a final volume of 400 µl. Incubation was carried out for 1 h at 56°C.

Semen stains were treated in the same way with the exception that preferential lysis (Gill et al. 1985) was carried out as follows: prior to lysis with proteinase K the extract was centrifuged at 13000 rpm for 15 min, the supernatant transferred to another tube (1.5 ml) and digested with proteinase K as described above. The sediment was washed 5 times in 0.9% NaCl by centrifugation for 15 min at 13000 rpm and then digested in 312.5 µl buffer II, 50 µl proteinase K, 12.5 µl 20% SDS and 25 µl 0.8 *M* DTT at 37°C overnight.

Extraction was carried out in phenol/chloroform/isoamylalcohol (24:24:1) and the DNA precipitated as described previously (Brinkmann et al. 1991).

DNA concentrations were estimated fluorimetrically and the presence of high molecular weight DNA was determined electrophoretically (Sambrook et al. 1989) as shown in Fig. 1. Restriction digestion was carried out with 30 U *Hinf*I (Gibco BRL, UK)/µg DNA overnight at 37°C. Completeness of digestion was determined electrophoretically. Some typical examples of undigested DNA, partial and complete digestion are shown diagrammatically in Fig. 2. Electrophoretic separation of DNA fragments, southern blot, probe labelling and hybridisation were carried out as previously described (Brinkmann et al 1991).

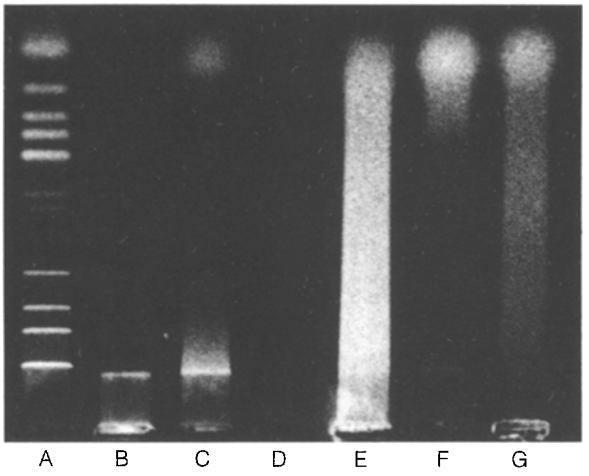

Fig. 1. Electrophoretic control for high molecular weight DNA after extraction from stains. DNA was visualised with ethidium bromide. Lane *A*, lambda phage DNA Standard (Drigest III, Pharmacia); lane *B*, high molecular weight DNA; lane *C-G*, various examples of high molecular weight DNA and partly degraded DNA

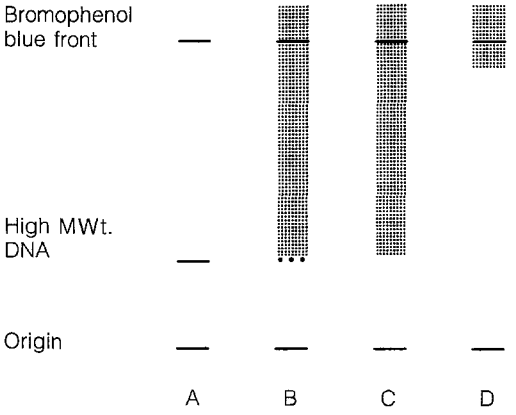

Fig. 2. Patterns of various stages of DNA digestion with *Hinf*I (courtesy of P. Gill CRSE, UK). Lane *A*, high molecular weight DNA; lane *B*, partially digested DNA with high molecular weight DNA; lane *C*, nearly complete digestion of DNA with minimal high molecular weight DNA content; lane *D*, complete digestion of DNA

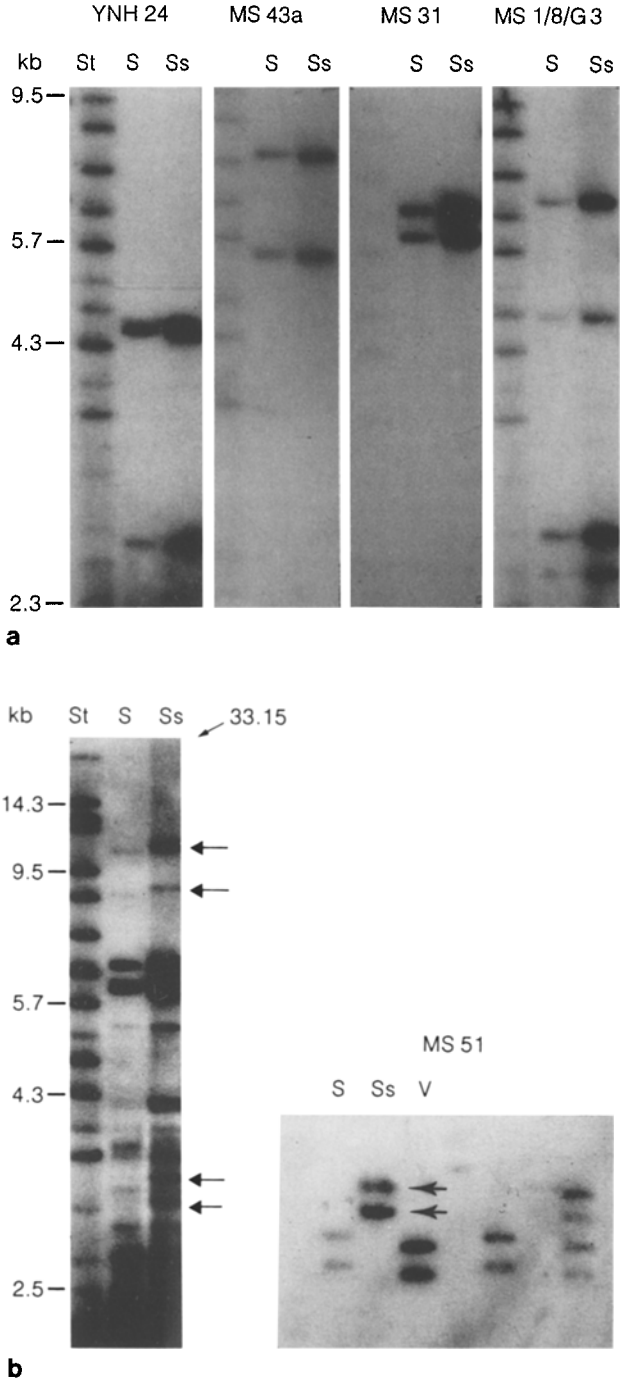

Fig. 3. a Autoradiographs of 6 SLP patterns in a case of violent rape. Restriction enzyme *Hinf*I. *St*, phage DNA Standard (Analytical marker DQ 1911, Promega); *S*, suspect blood DNA control; *Ss*, semen stain after preferential lysis. **b** Autoradiographs of MLP 33.15 and SLP MS 51 in the same case; *V*, Victim. Restriction enzyme: *Hinf*I. Extra bands and band position changes are marked with *arrows*

Results

Partial digest

In a rape case the sediment from a vaginal swab was analysed using 6 single locus probes. Three were rehybridised separately and 3 others as a mix (Fig. 3a). Absolute agreement was found between the sperm DNA and blood DNA from the suspect. However, rehybridisation with the MLP 33.15 revealed partial disagreement. Two extra bands and 2 band shifts could be seen (Fig. 3b, arrowed) which could have been interpreted as non-agreement.

Rehybridisation with the SLP MS51 (Fig. 3b), which is very sensitive to partial digest, also revealed disagreement between the sperm DNA and the suspect blood. The sperm bands were located in the same positions as the additional bands in the 33.15 pattern. – Depending on the degree of digestion MS51 can reveal 3 types of patterns (Fig. 4): lack of digest (A), partial digest (B) and complete digest (C). Since MS51 is derived from the MLP 33.15 and the positions of the deviant bands were identical in both probes, incomplete digestion was the most reasonable explanation for the occurrence of the 2 extra bands. It was also concluded that the strong shift in the higher molecular weight region could also be explained by the same phenomenon. – The digest control, which is routinely carried out, gave no indications of incomplete digestion. The results of the classical systems ABO, PGM and Gc gave no clear indications of the blood groups of the perpetrator.

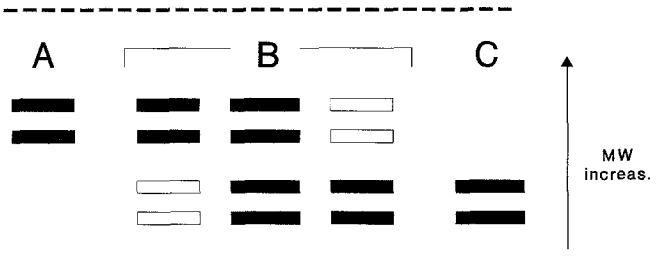

Fig. 4. Restriction patterns of the SLP MS51 (courtesy of P. Gill, CRSE, UK). Categ. *A:* poor restriction – only partial restriction band products present. Categ. *B:* incomplete restriction – partial restriction band products present in addition to fully restricted band products. Categ. *C:* fully restricted – only fully restricted band products present

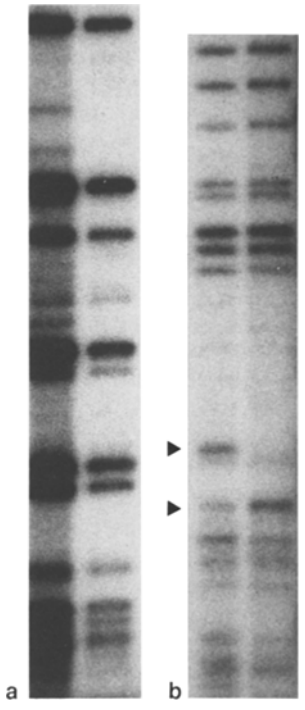

Fig. 5a, b. Autoradiographs showing 2 examples of band deficiencies using the multi locus probe 33.15/*Hinf*I. **a** Intermediate band deficiency between semen stain DNA *(right)* and blood DNA *(left)*. **b** Illogical changes in band intensity in some bands *(arrowed)* between semen stain DNA *(left)* and blood DNA *(right)*

Band loss

"Logical" band loss: In our experience when using MLP's such as 33.15 it is not unusual for the stain DNA to show bands of much weaker intensity or a lack of bands when compared to the blood control DNA (Fig. 5a). This is a deficiency problem caused by different DNA concentrations between blood control and a stain where only small amounts of DNA are present.

"Illogical" band loss: Another example of band pattern modification occurs where the band intensities are interchanged (Fig. 5b). In 2 patterns with otherwise comparable band intensities 2 bands showed reversal of intensity. Although both multi locus patterns could clearly be identified, changes in the band intensities were "illogical" because weak fragments in one sample were strongly expressed in the other and vice versa.

Co-electrophoresis of mixed samples

When only relatively small numbers of spermatozoa but numerous vaginal cells are present an alternative procedure is to carry out only a single lysis (1-step DNA extraction with proteinase K, SDS and DTT) to minimize the loss of sperm DNA. As expected, the autoradiograph of the DNA mixture showed a "mixed" band pattern (Fig. 6). This can either be complete which means that the female and the male bands can be completely differentiated (Fig. 6, MS43) or there is a partial overlap of 2 or more bands. The latter situation necessitates careful interpretation with regard to the male origin of the "mixed" fragment. In the case shown in Fig. 6, the inclusion of the shared fragment from YNH24 would have led to a frequency of 1:6250000 for the male pattern compared to only 1:625000 when not taken into account. For this calculation 4 SLP's were used (MS8, MS31, MS43 and YNH24).

Deficiency cases

In a murder case, the victim was raped and stabbed. The blood stain distribution pattern was not exclusively in accordance with the victims injuries. Comparison of the bloodstain with the band pattern of the suspect resulted

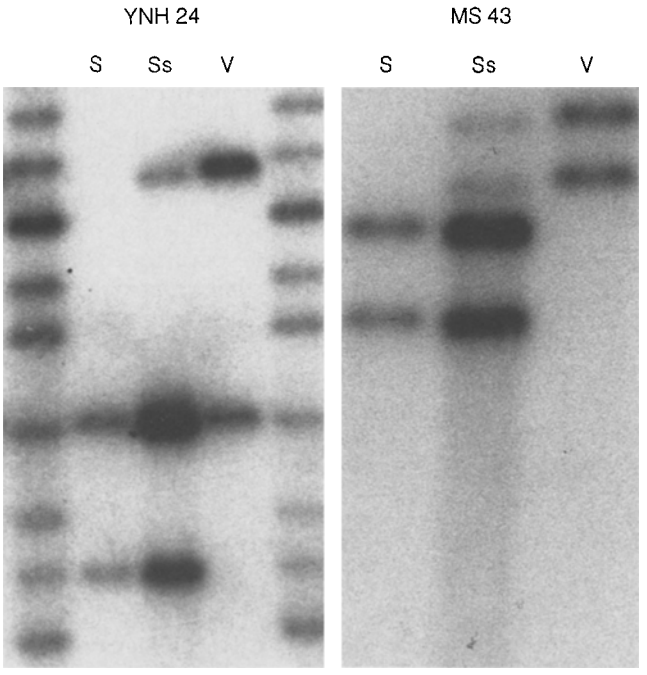

Fig. 6. Autoradiographs of 2 SLP's in a rape case with unseparated vaginal and semen components. Restriction enzyme *Hinf*I. *S,* suspect blood DNA control; *Ss,* semen cells contaminated with vaginal cells; *V,* victim blood DNA

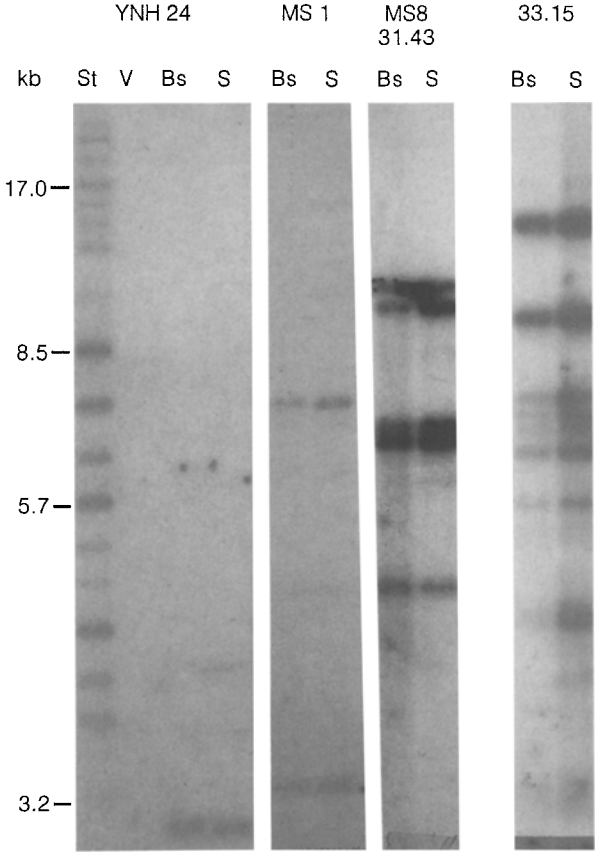

Fig. 7. Autoradiographs of 5 SLP's using a cocktail of 3 SLP's (MS 8, MS 31, MS 43) and 1 MLP (33.15). Restriction enzyme *Hinf*I. *St,* phage DNA Standard (analytical marker, Promega); *V,* tissue DNA from victim; no hybridisation because of degradation; *Bs,* bloodstain; *S,* suspect blood DNA control

in full agreement with 5 SLP's and one MLP (Fig. 7). The frequency of the 5 SLP's alone was $1:12.5 \cdot 10^6$ and in combination with classical systems $1:12.5 \cdot 10^{12}$. The cocktail with MS8, MS31 and MS43 was calculated with the fragment which has the highest frequency of all 5 fragments, taken into account the +/− 3 SD window. This calculation does not include the frequency of the MLP pattern. Since the victim had only been discovered several days after the crime had occurred the body was severely decomposed and the DNA heavily degraded.

Discussion

In most cases presented here only very little or no information could be obtained using classical systems with the exception of the bloodstain case. In contrast the DNA investigations were highly informative but a few problems should be pointed out. The susceptibility to partial digest is clearly greater for stain DNA and may be caused by methylation around or near the restriction site (Budowle et al. 1990). The problem of partial digestion has only been observed in casework with the MLP 33.15, and of course, with the SLP MS 51 which is especially sensitive to this process, but not with other SLP's. Shifts between blood DNA and sperm DNA have been described for MLP's and also SLP's and can be due to a variety of factors or a combination of factors. Methylation of restriction sites has been shown to differ between tissues and can result in differences in band patterns (Budowle et al. 1990; Washio et al. 1990) when DNA from blood and semen are compared. Different salt concentrations in samples resulting in star activity (Howard 1989), overloading of DNA and conformational differences (Grossmann 1989) have also been shown to affect band mobility. Although the latter has only been demonstrated in circular DNA it should not be discarded as a possible contributing factor. McNally et al.(1990) showed that considerable differences in electrophoretic migration could be found between fresh blood DNA and sperm stain DNA but not all bands were equally affected. An important consideration for the evaluation of the results of the VNTR analysis in these cases is that the shift is in the same direction and magnitude for all fragments affected. The maximum acceptable deviation between 2 fragments should therefore take into account the measurement error as well as a (natural) "shift" between fresh samples and stains.

One problem with the interpretation of MLP band patterns is that the variation in band intensity is generally independant of fragment size but dependent on the DNA concentration. This determines (inter alia), the number of bands in a given sample which can be visualised. It can easily be demonstrated that when the amount of DNA is reduced, as can occur in stains, the weaker bands are no longer visible and a band deficiency results. It is possible to take this 'logical' band deficiency into consideration when evaluating band patterns but the chance exists, at least theoretically, that another individual can possess most of the bands in the deficient pattern but not all and so cannot be excluded as a possi-

ble source. A biostatistical evaluation of these 2 possibilities can only be used to estimate which is more likely.

An "illogical" band deficiency cannot be so easily explained. It can be argued that the change in intensity and position of bands could possibly be due to partial digest caused by methylation of the restriction site and subsequent appearance of a band or bands in other positions. This has however not yet been adequately investigated.

In rape cases where only small amounts of spermatozoa are found it could be useful to dispense with preferential lysis to avoid further loss of DNA but the results could be adversely affected by the disproportionate amounts of vaginal and sperm DNA. Analysis of band patterns from mixtures could be reliably carried out by comparison of the corresponding band patterns from victim and suspect blood samples. If a band overlap is found (within one system) between victim and suspect, the common band should not be included in the biostatistical calculation of frequency. Although the overlapping band can sometimes be more intense this is not always the case. It is not sufficient evidence that this band should be assigned to the suspect and should not be included in the statistical calculation.

One special characteristic of investigations with DNA can be seen in deficiency cases. The high degree of individuality attained using a combination of several hypervariable single locus probes means that a match between an unknown stain and suspect gives such a high probability of identity that the possibility that the stain could have originated from another person is practically ruled out. This is particularly useful when no DNA can be obtained from the victim for comparative purposes due to various reasons such as decomposition or degradation as shown in the deficiency case described here.

If only highly degraded DNA can be isolated it is unlikely that conclusive results will be obtained from SLP or MLP analysis. In this case one possibility is the use of PCR-AMPFLP's or HLA DQα – which does not rely on the presence of high molecular weight DNA. In cases of multiple rape it is also possible using PCR-AMPFLP's to distinguish between different sperm populations in a mixture and to assign the bands to more than one perpetrator. In this case a combination of several AMPFLP systems could prove to be a solution in the future (Kasai et al. 1990; Budowle et al. 1991). However, extensive population studies and the minimalisation of artefacts are necessary before these systems can be established and used in stain investigations. In consideration of recent developments, some material from each stain case should be retained for future investigations.

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