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A common disinfectant used in condom processing inhibits endonuclease digestion of sperm DNA

S. Fadda[1], S. Pelotti[2], and G. Pappalardo[2]

[1]Istituto di Clinica Neurologica, Laboratorio di Genetica, Università di Bologna, Via Foscolo 7, I-40123 Bologna, Italy
[2]Istituto di Medicina Legale, Università di Bologna, Via Irnerio 49, I-40126 Bologna, Italy

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Summary. DNA recovered from a condom found at a crime scene appeared undigestible with restriction enzymes, preventing characterization by Southern blot and polymorphic probe hybridization. Several chemical substances used in the processing and treatment of condoms were tested for inhibitory action of restriction enzymes. In particular dibenzalkonium chloride appeared to promote enzyme inhibition at very low concentrations. The effectiveness of treatments to restore cleavage of sample DNA in the presence of such contaminants is discussed.

Key words: Sperm DNA – Endonuclease digestion – Dibenzalkonium chloride

Zusammenfassung. DNA, welche von einem an einem Tatort gefundenen Kondom gewonnen wurde, erwies sich als unverdaulich mit Restriktionsenzymen. Hierdurch wurde die Charakterisierung mit Southern Blot und Hybridisierung mit einer polymorphen Probe verhindert. Mehrere chemische Substanzen, welche bei der Herstellung und Behandlung der Kondome benutzt werden, wurden auf ihre inhibitorischen Eigenschaften gegenüber Restriktionsenzymen getestet. Insbesondere erwies sich Dibenzalkoniumchlorid als wirksam, die Enzyminhibition bei sehr niedrigen Konzentrationen zu verursachen. Die Effektivität von Maßnahmen, um die Schneidbarkeit der Proben-DNA bei Gegenwart solcher Kontaminierungen wieder herzustellen, wird diskutiert.

Schlüsselwörter: Sperma-DNA – Endonucleasen-Verdau – Dibenzalkoniumchlorid

Introduction

In forensic practice it would be helpful to evaluate the probability of achieving a successful DNA analysis on the basis of sample amount and carrier material. Low yields and partial restriction are 2 major causes of failure in the characterization of DNA recovered from semen samples [1]. Different carriers appear to significantly affect the quality and quantity of DNA extracted from bloodstains [2]. In many cases recovery can be improved by means of appropriate experimental procedures. Unknown substances interfering with the ability to analyse DNA were also postulated in a criminal case where seminal fluid was recovered from inside a condom. This case spurred the investigations reported in this paper. Briefly, about 200 µl seminal fluid was recovered from a condom found at a crime scene. The semen showed no sign of degradation nor contamination by dust, blood or vaginal cells upon microscopic examination and electrophoresis of DNA extract. However, DNA was extracted with relatively low yields; digestion could not be accomplished even with tenfold amounts of restriction endonucleases despite long incubation and addition of spermidine trichloride. The transfer of fragments from agarose was also poor. Microdialysis of the sample allowed prompt digestion and transfer, so that Southern blot characterization by 3′HVR [3] could be carried out. Amplification of DNA with DQ α and DP β primers was possible before dialysis, allowing HLA typing at the DNA level. These problems were not found in the DNA controls extracted from sperm and blood with identical procedures.

The tentative identification of relevant contaminants is described in connection with the procedures that appear to eliminate their effect.

Materials and methods

Sperm DNA isolation. Semen samples were obtained from 2 donors in sterile plastic containers and stored frozen until extracted. Several postcoital samples from a 3rd male donor and partner were also obtained in glass containers and preliminary lysis of female cells was achieved following the procedure described elsewhere [4]. Subsequently all the samples were processed following the method of Gill et al. [5] for pure semen. Briefly, samples were extracted twice with 1 vol. phenol/chloroform (pretreated as in Maniatis et al. [6]). DNA was precipitated by addition of sodium acetate (final concentration 0.3 *M*) and 2.5 vol. chilled absolute ethanol, stored overnight at −20°C and centrifuged at 15000 *g*, 4°C for 30 min. The supernatant was decanted, the pellet was

Offprint requests to: G. Pappalardo

gently air-dried and resuspended in TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.5).

A final microdialysis through a Millipore filter (0.05 μm) as described by Werrett et al. [1], was carried out when indicated in the text after treatment with various chemical substances before and/or after endonuclease restriction.

Blood DNA isolation. DNA was extracted following the procedure of Maniatis et al. [6] with modifications. Microdialysis was applied to samples treated with chemical substances when indicated in the text.

Tests on the condom washing mixture and on its chemical components. Commercially available condoms (Hatů, Italy) were rinsed with 100 μl double distilled sterile water and this rinse was added to DNA samples immediately prior to digestion with the restriction enzymes TaqI and PvuII. The following substances used in condom processing and treatment with the indicated function were kindly provided by Hatů, Italy, and were assayed at the concentrations reported below:

1) Colloidal sulphur: used in rubber vulcanization, provided as powder, assayed at 0.3 mg/ml water or less.
2) Vulkacit LDA: zinc diethyl carbamate (Bayer), used in rubber production, provided as powder, assayed at 0.16 mg/ml water.
3) Vulkacit PXN: zinc pentamethyl carbamate (Bayer), used in rubber production, provided as powder, assayed at 0.3 mg/ml water.
4) Biosorb: starch plus epichloridrine preserver (Ethicon), used as talc, provided as powder, assayed at 0.67 mg/ml water.
5) Lycotex: calcium carbonate plus aluminum stearate (Wilfrid Smith, UK), used as talc, provided as powder, assayed at 1 mg/ml water.
6) Vulkanox BKF: 22-methylen-bis-4-methylbutylphenol (Bayer), used as an antioxidant factor, provided as powder, assayed at 0.5 mg/ml water.
7) Emulan: fatty acid ethoxilate (BASF) used as disinfectant, provided as powder, assayed at 6.7 mg/ml water.
8) B.A.C.: dibenzalkonium chloride (Henkel), used as disinfectant, provided as a 50% solution in water, assayed at 10% or less in water.
9) Dioctylsulphosuccinate sodium salt: used as spermicide, provided as a polyglycol solution, assayed at 20% in water.
10) Silicon oil: (Bayer), used as lubricant, provided as a viscous liquid, assayed as such.

DNA restriction, electrophoresis, blotting and characterization. Aliquots (2–5 μg) of DNA were digested for 1–2 hours with TaqI and PvuII restriction endonucleases (Boehringer Mannheim), in a volume of 25 μl incubation buffer (up to 30 μl for more diluted DNA samples), at 1 unit or 2 units per μg DNA respectively, following the manufacturer instructions, in the presence or absence of test solutions. Aliquots of the digests (1 or 2 μl) were electrophoresed and inspected to assess the extent of digestion. Some of the undigested samples were dialyzed as described [1] or re-extracted, and then redigested as above. The digests were loaded on a 0.8% agarose gel and electrophoresed in TAE buffer [6], at 20 V for 18 hrs. The gel was stained in ethidium bromide and photographed under UV light. Some of the gels were blotted onto Hybond N+ membrane (Amersham) with the alkali procedure [7] and the filter hybridized to the probe 3'HVR [3] radioactively labeled with ^{32}P using the random priming technique [8]. Hybridization and washings were done according to the procedure of Church and Gilbert [9].

Results

As the sperm carrier in the case sample was suspected of having played a role in the modification of DNA or of enzymes, a new condom was washed with sterile water and the suspension obtained was tested for its ability to prevent sperm DNA digestion. As the mixture proved to inhibit restriction very effectively, its components were tested separately to search for the non-extractable dialysable substance that conferred the inhibition ability. For the substances inhibiting endonuclease activity at high concentration, 2 procedures were followed: either extraction or dialysis were applied to samples where digestion was incomplete and digestion was repeated, or both treatment and decontamination were applied before digestion. The results were identical, regardless of the procedure, and were pooled together.

For all substances (at the maximum tested dosages, see Materials and Methods), as well as for the case sample and the carrier washing, a qualitative evaluation of inhibitory action and its reversal is reported in Table 1. Examples are shown in Fig. 1: inhibitory or non-inhibitory behaviour was always clearcut.

Three substances appeared to inhibit restriction at the tested dosages: dioctyl sulphosuccinate, sulphur an benzalkonium chloride. Dioctyl sulphosuccinate is phenol

Table 1. DNA restriction inhibition by several condom substances and its reversal by physicochemical procedures

Tested samples	Tested substance	Restriction inhibition	Inhibition removal		
			Extraction	Dialysis	Spermidine
Casework sperm DNA	Unknown	++	−	+	+−
Control sperm DNA	Condom washing	++	−	+	+−
Control sperm DNA	Sulphur	++	−	+	/
Control sperm DNA	Vulkacit LDA	−			
Control sperm DNA	Vulkacit PXN	−			
Control sperm DNA	Biosorb	−			
Control sperm DNA	Lycotex	−			
Control sperm DNA	Vulkanox BKF	−			
Control sperm DNA	Emulan	−			
Control sperm DNA	Benzalkonium chloride	++	−	+	+−
Control sperm DNA	Dioctylsulphosuccinate	++	+	−	−
Control sperm DNA	Silicon oil	−			

− No inhibition; + inhibition; ++ strong inhibition; +− moderate inhibition; / not tested

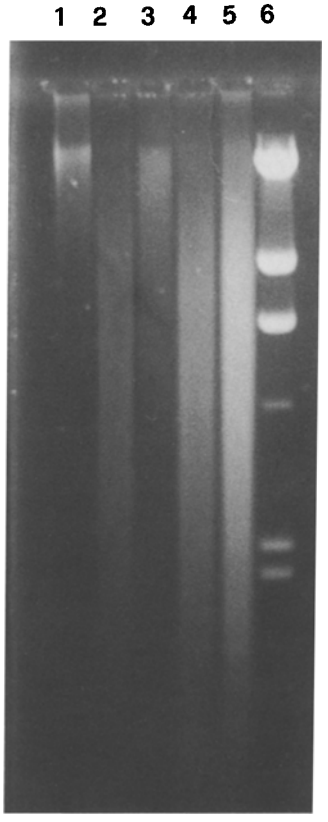

Fig. 1. Ethidium bromide stained agarose gel to show the effect of some chemical substances used in the treatment of a sperm stain carrier (condom) upon restriction endonuclease digestion of sperm DNA. Lane *1*, control DNA (no enzyme, no test substance); lane *2*, digested control DNA (TaqI enzyme, no test substance); lane *3*, DNA digested in the presence of dioctyl sulphosuccinate; lane *4*, DNA digested in the presence of Emulan; lane *5*, DNA digested in the presence of silicon oil; lane *6*, HindIII digested λ DNA

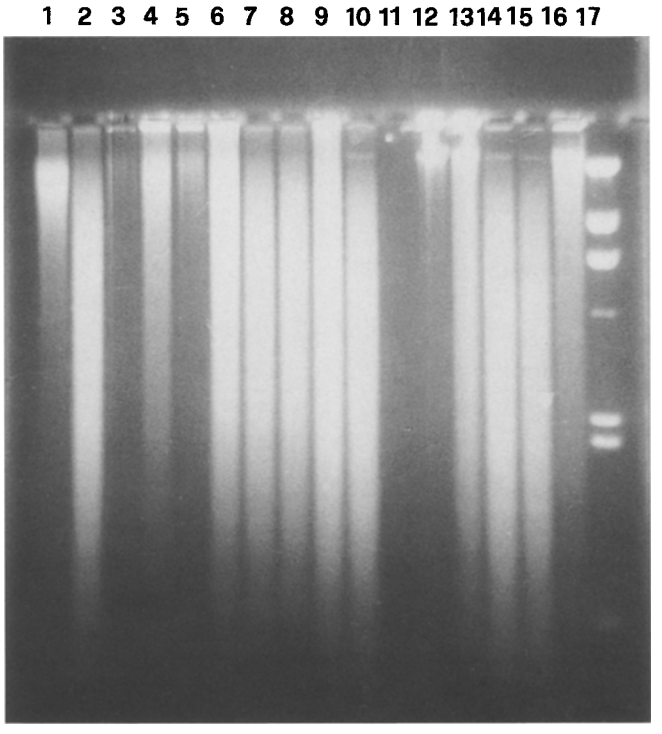

Fig. 2. Ethidium bromide stained agarose gel to show the effect of BAC upon restriction endonuclease activity and restoration of enzyme activity by dialytic procedures. PvuII digestion, sperm DNA: lane *1*, undigested, untreated DNA; lane *2*, digested, untreated DNA; lane *3* digested, 1% BAC treated DNA was lost during manipulation; lane *4*, digested, 0.5% BAC treated DNA; lane *5*, as lane 3, 1% BAC treated DNA was recovered; lane *6*, 0.25% BAC treated DNA; lane *7*, *8* as lane 4, 6, plus dialysis and redigestion; lane *9*, as lane 6. TaqI digestion blood DNA: lane *10*, control digested DNA, no test substance, no dialysis, (high molecular weight band may be due to the use of limiting amounts of restriction enzymes); lane *11*, DNA digested in the presence of 1% BAC: DNA was lost during the transfers; lane *12*, DNA digested in the presence of 0.4% BAC; lane *13*, DNA digested in the presence of 0.2% BAC; lane *14*, as lane 12, plus dialysis and redigestion; lane *15*, as lane 13 plus dialysis; lane *16*, DNA digested in the presence of 0.3% BAC; lane *17*, HindIII digested λ DNA

extractable and should not be a contaminant of extracted DNA. Moreover it is used as spermicide in a more expensive make of condoms. The other 2 substances were not phenol extractable, but were successfully eliminated by dialysis, and were therefore tested at lower concentrations: sulphur appeared to be effective at concentrations above 30 ng/ml and benzalkonium chloride above 0.1%.

DNA treated with high concentrations of BAC became viscous and did not enter the exclusion point of the gels (Fig. 2). Spermidine (4 m*M*) has been claimed to facilitate digestion [5], but did not affect it significantly in this system (results not shown).

The effects of threshold concentrations of these substances were also tested with DNA extracted from blood and digested with the same restriction enzymes. No difference was observed between enzymes or with respect to sperm extracted DNA (Table 2, Fig. 2).

Discussion

Large differences in the numbers of DNA-containing cells are often responsible for the extensive variation in DNA yields reported in forensic practice. However, reduced recovery and poor quality of DNA can also originate from unknown contaminants contributed by sample carriers. This problem has been investigated for bloodstain carriers by Prinz and Bergaus [2]. Having experienced similar problems with sperm recovered from a condom, we tried to identify contaminating substances in the carrier washing mixture. Identification could be the first step towards the understanding of the mode of action and the adoption of proper elimination procedures.

A battery of chemical substances are used in the processing of condoms. However, most of them (7 out 10) did not affect restriction endonucleases at all, even at high concentrations. Few substances (3 out of 10) showed clearcut inhibition: one of those could be excluded on the basis of its solvent affinities (dioctylsulphosuccinate appeared to be eliminated by phenol extraction), and

Table 2. Determination of threshold concentrations for the substances that inhibit restriction enzymes, but are removed by dialysis

Sample	Tested substance								
	Benzalkonium chloride (% concentration)						Sulphur (ng/ml)		
	10	1	0.4	0.2	0.1	0.01	300	30	3
Sperm DNA									
– *Taq*I digest	+	+	+	+	–	–	+	+	–
– *Pvu*II digest	/	+	+	+	–	–	/	/	/
Blood DNA									
– *Taq*I digest	+	+	+	+	–	–	+	+	–
– *Pvu*II digest	/	+	+	+	–	–	/	/	/

+ inhibition; – no inhibition; / not tested

another because its free form should not be present in the finished product (sulphur, used in the early phase of condom production).

The remaining contaminant was the antiseptic benzalkonium chloride, a cationic detergent which precipitates with anionic detergents (Merck Index).

Interference with DNA solubility is a property of other organic derivatives of quaternary ammonium, exhibiting remarkable similarities with BAC in their structure and charge: precipitation by CTAB (cetyl trimethyl ammonium bromide) and TTAB (tetradecyl trimethyl ammonium bromide) has been proposed for rapid DNA purification, avoiding usual phase separations [10, 11].

Similarly, BAC can precipitate DNA at low ionic strengths, (TE 0.1–0.01 *M* buffer [6]) as under our experimental conditions or in 0.6 *M* NaCl, but is ineffective at higher ionic strength (1.2 *M* NaCl, results not shown).

BAC has visible effects upon DNA, whether extracted from sperm or blood. Endonuclease digestion is strongly inhibited in its presence and 4 m*M* spermidine does not improve the situation. However, BAC does not seem to interfere with DNA duplication (in our casework PCR amplification was successfully performed) and is promptly removed by microdialysis.

Recent evidence indicates [12] that TMAC (tetramethylammonium chloride), another quaternary ammonium salt chemically similar to BAC, can act as an amplification enhancer, improving the specificity of PCR reactions without any interference with Taq polymerase activity at the effective concentrations.

It is interesting to note that BAC is not limited to condoms in its use, being commercially available as a disinfectant under a variety of names (Germinol, Roccal, Zephirol etc; Merck Index), which are major components of disinfectant blends (Citrosil, Bialcol etc.) for home and hospital first aid. Therefore BAC is likely to be found on more common trace carriers and also in apparently clean samples.

Simplified protocols, omitting the laborious dialysis step, were recently proposed in crime investigations for mass screening of DNA profiles [13] and were successfully applied to blood samples collected under controlled conditions. They may not be as suitable for forensic samples found on unreliable carriers. Our casework shows that dialysis may be necessary, as an early or late step in purification procedure, to obtain enzymatic digestion of DNA.

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