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MtDNA typing of single-sperm cells isolated by micromanipulation

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ABSTRACT

Some sexual assault crimes constitute a problem for the legal institutions confronted with the DNA analysis of such cases. Often, sperm cells are found in the victim's vaginal tract during medical examination but their successful genotyping is compromised by the huge excess of the victim's epithelial cells as well as by the degradation of genomic DNA present in sperm cells as a consequence of female immune response.

Mitochondrial DNA present in the mid-piece of sperm cells might be useful in some specific cases in order to differentiate the donors of a semen sample. The high number of copies per cell and its circular nature that may confer some protection from the action of exonucleases make it more suitable for cases where few cells are available and/or the DNA is degraded.

We have developed a novel strategy for typing mtDNA from single-sperm cells. Specific amplification of male mitochondrial DNA is ensured by use of sequence specific primers designed on the basis of mitochondrial single nucleotide polymorphisms existent throughout the control region. The strategy was applied to single-sperm cells isolated by micromanipulation from slides smeared with vaginal swabs taken immediately after sexual intercourse of voluntary couples. After sequencing the PCR products, it was possible to obtain a match between the DNA sequence from the buccal swab and the DNA sequence of the single sperm-cell, for each voluntary man. With this new strategy, the problem of contamination with DNA from the victim observed when using universal primers was completely overtaken. This method will probably allow the resolution of multiple-rapist crimes, where the collected sperm cells can be separately typed.

1. Introduction

Sexual assault is one of the most frequent crimes all over the world. After a rape, traces of the aggressor can be found on towels, sheets, clothes and other surfaces, as well as on the victim's hair and skin. However, one of the most prolific sources of rapist biological material are the sperm cells that the victim might still carry in her reproductive tract and/or anal epithelium [1].

The standard genotyping techniques (as short tandem repeat (STR)) are based on the amplification of small DNA fragments. The sequences on the template DNA used for the primer annealing are conserved among the individuals. For this reason the primers amplify any DNA sample present on a mixture and they are also not able to discriminate between male or female DNA. This represents a major problem which compromises the analysis of vaginal swabs collected after sexual assault crimes as they contain up to 167 times more female cells than sperm cells and so the female nuclear DNA is preferentially amplified [2].

Different approaches have been proposed attempting the separation of male and female cellular fractions or DNA: differential lysis [3], fluorescence-activated cell sorting (FACS) [4], antibody conjugated magnetic bead capture system [5], laser microdissection [2] and microchip-based separation [1,6] are the most widely used techniques. From these, laser microdissection has been better proving its efficacy in the presence of samples with low number of sperms mixed with female/victims' cells [2]. However the costs associated with the mentioned technique are extremely high, being difficult for common laboratories to acquire the necessary equipment. Another problem associated with STR genotyping is that, with time since intercourse, female DNA from lysed cells can adhere to the sperm membrane and be internalized compromising the specific amplification and analysis of male STR fragments [7,8]. One alternative strategy to analyse male DNA avoiding the contamination with female DNA is the Y-STR analysis. Although it has been successfully applied in various crucial situations, this procedure is very prone to degradation of the template DNA and does not allow typing of a single-cell [9].

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Some factors appear to influence the ability to amplify DNA markers in degrading material like cellular location, the transcriptional activity and the gene copy number [10]. For cases where few cells are available and/or the DNA is found degraded, mtDNA might be valuable given that in a single cell there exists a high number of mtDNA copies and its circular nature may confer protection against the action of exonucleases [11]. Sequencing the mitochondrial control region (also known as D-Loop) is internationally accepted as an efficient tool for typing extremely difficult biological forensic traces [12–14].

The work here presented uses the micromanipulation technique [15] to isolate sperm cells from a glass slide smeared with a vaginal swab and to transfer them to a tube for DNA extraction. Besides the physical separation from the male and female cells, sequence specific primers (SSP) for the man were used to assure that the DNA from the woman would not be co-amplified. The primer design was based on the mitochondrial DNA (mtDNA) haplotype differences between the man and woman determined after mtDNA analysis of buccal swabs. This procedure allows the characterization of the male mitotype from a single-sperm cell present in a vaginal swab.

2. Materials and methods

2.1. Anti-contamination practice

To minimise the occurrence of contamination, the guidelines given by the European DNA profiling (EDNAP) group in 2001 were followed [16].

2.2. Samples

All the samples were obtained under informed consent from 5 male and female volunteer couples. The samples consisting of vaginal swabs were collected immediately after sexual intercourse according to standard procedures and air-dried before further usage. Buccal swabs were obtained from all the volunteers and similarly air-dried before usage.

2.3. DNA Isolation from buccal swabs

The DNA from the buccal swabs was isolated using the *QIAamp® DNA Mini Kit* (QIAGEN, Germany) following the recommended protocol and an elution volume of 200 μl.

2.4. Amplification of D-loop region by Polymerase Chain Reaction (PCR) using DNA isolated from buccal swabs

In order to type the D-loop region of the mtDNA from all the participants in the study, 25 ηg DNA isolated from buccal swabs was amplified using standard primers L15869 and H719 (Table 1) (Thermo, Germany). PCR was carried out in a total volume of 25 μl consisting of 1×PCR buffer (QIAGEN, Germany), 200 μM of each dinucleotide triphosphate (dNTP) (PeqLab, Germany), 0.2 μM of each primer (Roth, Germany), and 1U of Hotstar Taq DNA Polymerase (QIAGEN, Germany). Amplification reaction was performed on a Biozym PTC-225 Tetrad thermal cycler (MJ Research, USA) with an initial 14 min incubation period at 95 °C followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1.5 min. The program also included a final elongation step at 72 °C for 7 min. In order to test PCR reagents for contamination with external DNA, non-template controls (negative controls) were performed in all reactions. After amplification, the success of the reaction was confirmed by electrophoresis in a 1.0% agarose gel (Roth, Germany) in TBE buffer [17]. For that 4 μl of each PCR product were loaded and run for 30 min at a constant voltage of 150 V. To confirm the molecular weight of the PCR product obtained, a 100 bp DNA-ladder extended (Roth, Germany) was also loaded in the electrophoresis gel.

Table 1
Primers used to amplify and to sequence the mitochondrial control (D-loop) region using DNA isolated from buccal swabs.

Amplification region	Direction	Primers	Sequence
D-loop region	Forward	L15869	5′-AAAATACTCAAATGGGCCTGTC-3′
	Reverse	H719	5′-CGTGGTGATTTAGAGGGTGAAC-3′
HVI	Forward	L15996	5′-CCA CCA TTA GCA CCC AAA GC-3′
	Reverse	H16401	5′-TGA TTT CAC GGA GGA TGG TG-3′
HVII	Forward	L27	5′-CAG GTC TAT CAC CCT ATT AAC-3′
	Reverse	H411	5′-ACT GTT AAA AGT GCA TAC CG-3′

2.5. Purification of PCR products

When successful amplification was observed on the gel electrophoresis described above, the remaining PCR products were purified with the QIAquick® spin PCR purification kit (QIAGEN, Germany) and eluted with 50 μl of the corresponding elution buffer.

2.6. Cycle sequencing reaction

According to the standard recommendations both mtDNA strands were analysed [16]. Sequencing reactions were made using BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, USA) in a 10 μl reaction volume according to manufacturer's protocol. The reaction was performed on a PTC-225 Tetrad thermal cycler (MJ Research, USA) and involved an initial denaturation step at 96 °C for 30 s and 25 cycles of denaturation at 96 °C for 30 s, annealing at 48 °C for 15 s and extension at 60 °C for 4 min. The primers used are listed in Table 1. The removal of excess dye-deoxy terminators was accomplished with DyeEx 2.0 Spin Kit (QIAGEN, Germany) according to the manufacturer's instructions. The automated DNA sequencing was carried out on a 3730 DNA Analyzer (Applied Biosystems, USA) using the Performance Optimised Polymer POP 7 (Applied Biosystems, USA). The denaturated samples were injected into a 36 cm capillary and the electrophoresis was run with a rapid sequencing module. The analysis of mtDNA sequencing results was performed using Sequencing Analysis v5.2 software (Applied Biosystems, USA). The sequences of both strands were aligned and compared with the rCRS [18] using the Clone Manager Suite v.6 software (Scientific & Educational Software, USA). The analysis was mainly focused on single nucleotide polymorphisms (SNPs) existent between the man and the woman as this was considered a crucial step for later design of SSPs.

2.7. Isolation of single-sperm cells by micromanipulation

The vaginal swabs were immersed in a 1,5 ml Eppendorf tube containing 200 μl of sterilised water for 1 h. During that time the swabs were pressed against the tube's walls to facilitate the liberation of sperm cells. Subsequently, the liquid was spread over a glass slide (Engelbrecht, Germany) and air dried. Sperm cells were identified under a DMIRB/E inverted microscope (Leica Microsystems, Germany) and a 400-fold magnification. In order to diminish contamination only sperm cells without direct contact to female epithelial cells were selected for capture. In order to facilitate the liberation and capture, a drop of sterilised water was put over the identified sperm cell. The cells immersed on the water drop were captured using a mechanical micromanipulator