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.

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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].
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 ng 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 dinitucleotide 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 was 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.

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 v6 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 SSP.

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 DMRB/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.

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

<table>
<thead>
<tr>
<th>Amplification region</th>
<th>Direction</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-loop region</td>
<td>Forward</td>
<td>L15869</td>
<td>5’-AAATATCCTAAATGGCGGTGTC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>H719</td>
<td>5’-CGTGGCTTATAGGGGATGAC-3’</td>
</tr>
<tr>
<td>HVI</td>
<td>Forward</td>
<td>L15996</td>
<td>5’-CCA CCA TTA GCA CCC AAA GC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>H16401</td>
<td>5’-TGA TTT CAC GCA GGA TGG TG-3’</td>
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<tr>
<td>HVII</td>
<td>Forward</td>
<td>L27</td>
<td>5’-CAG GTC TAT CAC CCA AT ATT AAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>H411</td>
<td>5’-ACT GCT AAT ACT GCA TAC CG-3’</td>
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Table 2

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<tr>
<td>16093</td>
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Table 2. SNPs profile for the region between mDNA-positions 15869 and 340 (covering HVI and HVII regions) of the 10 individuals (5 couples) that participated in this study and of the technician responsible for the analysis. Profiles were obtained after amplification of mDNA isolated from buccal swabs and sequencing using the primers listed in Table 1. The positions used to anchor the SSP (listed in Table 3) for each couple are shaded in grey while the SNPs analysed using that same SSP (together with the pairing primer listed in Table 3) are shaded in yellow.

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Leica Microsystems, Germany) and a CellTram™ Oil (Eppendorf, Germany) device. For the micromanipulation, we used borosilicate-glass capillaries (Hilgenberg, Germany) with a diameter opening of approximately 1.0 mm and prepared on a vertical Needle Pipette Puller (David Kopf Instruments, USA). Both capillary and connection tube were filled with liquid paraffin (Sigma Aldrich, USA). After micromanipulation, each spermatozoon was put on a 0.2 ml tube containing 15 µl of ATL buffer from the QIAamp® DNA Investigator Kit (QIAGEN, Germany).

2.8. DNA Isolation from single-sperm cells

The DNA present in single-sperm cells was isolated using the QIAamp® DNA Investigator Kit (QIAGEN, Germany) according to the manufacturer’s protocols and performing the final elution step with 25 µl of the appropriate buffer.

2.9. Design of sequence specific primers (SSP) and PCR optimization

SSP used at this stage were designed on the basis of the SNPs previously determined by sequencing the control region using DNA isolated from buccal swabs (Section 2.6). For each couple a SSP was designed to hybridize specifically with the man’s mtDNA. For that, the 3’ of the SSP was anchored on a SNP that differentiate the man’s mt-haplotype from the one of the woman. Primer design was done using Oligo 6, v6.7.1.0 software (Molecular Biology Insights, Inc., USA) and it was carefully made in order to avoid primer dimers and high Tm difference between SSP and the corresponding non-specific primer. The best annealing temperature (Ta) regarding specificity and efficiency for each primer pair was identified by Ta gradient PCR. Using the optimal Ta determined previously, all the SSP were tested on PCRs containing a mixture of equal amounts of female and male DNA.

2.10. PCR-SSP using DNA from single-sperm cells

For all couples the amplification of DNA isolated from single-sperm cells was performed in two steps using a boosted PCR strategy. The whole DNA was used in the first PCR round. This reaction was carried out in a total volume of 50 µl using the same conditions as previously described for buccal swabs. This time primers used were one SSP and one standard primer as shown in Table 3 (Thermo and Invitrogen, Germany). After the first amplification, a second PCR round was performed with the same primers. The successfully amplified PCR products were purified and sequenced as described previously for buccal swabs, in order to confirm the specificity of the whole procedure. In addition, for couple #5, an alternative nested PCR strategy was also applied. In a first round of amplification, standard primers L15869 and H719 were used. In a second round, we used the primers listed in Table 3 for the respective couple.

For all PCR reactions non-template controls were performed. In those involving small amounts of DNA, positive controls were also used. These consisted of 3.5 pg (corresponding to 1 human haploid cell) of DNA isolated from buccal swabs.

3. Results

3.1. MtDNA typing from buccal swabs

For all participants, including the responsible technician, the mtDNA control region was sequenced. For each couple several differences in the sequence were observed between the male and the female partner (Table 2).

3.2. SSP design and test using a mixture of DNAs from buccal swabs from both couple partners

Based on the sequence differences identified it was always possible to chose one SNP to anchor one SSP (forward or reverse). For example, the chosen SNP of couple #2 was 16398A. As this SNP is located between HVI and HVII, the designed reverse SSP used together with the L15996 primer allowed the amplification of the complete HVI region as well as the analysis of the respective sequence. The SSP designed for the 5 couples and the corresponding pairs are listed in Table 3.

For each primer pair it was possible to determine an appropriate Ta (Table 3) ensuring an efficient and specific amplification of the man’s DNA and no amplification of woman’s DNA. This was primarily confirmed by PCR using separately male and female DNA (Fig. 1). Additionally, the same primers were also used on PCRs containing a mixture of both male and female genetic material. After sequencing the PCR products, the sequences obtained showed exclusively the male mtotype of each couple (Fig. 2).

![Fig. 1](image_url)

**Fig. 1.** 1.0% Agarose gel showing PCR products (194bps in size) obtained using primers L16249C (forward-SSP) and H16401. Reactions were performed at different Ta using as template DNA isolated from buccal swabs from the man or from the woman constituting couple #1.
3.3. Isolation of single-sperm cells by micromanipulation

All of the slides smeared with vaginal swabs showed a big number of sperm cells. It was often observed that 1 or 2 min after covering the cell with water, the cell started to liberate by itself from the slide. When that did not happen, scratching of the slide with the micromanipulation capillary helped releasing the cell.

3.4. MtDNA typing of single-sperm cells

For any of the strategies followed (boosted PCR and nested PCR) after a first round of amplification with SSP no products were observed. Products visible on gel electrophoresis were obtained only after the second round of amplification.

Using the boosted PCR strategy satisfying amplification products were obtained leading to good sequencing results on approximately 75% of the single-cells isolated by micromanipulation (Fig. 3). For each cell, the complete haplotype of the man was obtained. There were no background signals belonging to the women’s variants (Table 4).

The nested PCR strategy additionally used for couple #5 resulted on a successfully amplification product and the sequence analysis revealed also the mt-haplotype from the man (data not shown).

4. Discussion

Forensic laboratories have invested much effort in developing techniques for the identification of rapists using vaginal fluid of sexual assault victims. The major problems faced by the existing methods are associated with the difficulty of separating sperm cells from the female cells and with the existence of cell-free DNA in the vaginal fluid as a consequence of tissue damage and fragmentation of the different cell types present in the vagina as epithelial cells, neutrophils and macrophages. This seems to be of crucial importance once that mature spermatozoa are able to internalize external DNA [7]. This process seems to be mediated by a DNA-binding protein (DBP) of 30–35 kDa in association with CD4 receptors on the surface of spermatozoa [8, 19]. It is not dependent on any DNA sequence and seems to involve reversible ionic interactions between DNA and the receptor [8, 20, 21]. Once internalized the female DNA can interact with the sperm DNA causing activation of sperm nucleases that cleave sperm DNA, eventually leading to apoptosis [7, 22]. Over 65% of sperm cells internalize DNA within 2 h of incubation in the absence of seminal fluid. Interestingly, an inhibitory factor (IF-1) present in the seminal plasma of most of the species and also on the surface of spermatozoa from lower animals that lack seminal plasma, has been found to block the internalization process [19]. In the vaginal milieu, with time since intercourse, the protective/inhibitory effect of IF-1 gradually decreases (eventually by dilution), favouring the DNA internalization by the sperm cells and the associated consequences. This might also be pointed out as a reason why the autosomal STR analysis is greatly hampered when samples are collected more than 48 h after intercourse [9].

The present work introduces a protocol that combines an effective physical separation of male cells from female cells in the vaginal fluid with an individual-specific typing approach. The procedure employs the more abundant and eventually more resistant to exonuclease degradation mitochondrial genome and offers highly reliable results on single cell typing of the respective control region.

The SSPs used in our typing strategy were anchored on mtDNA SNP differences observed between female and male from each of

![Single-Sperm Cells](image)

Fig. 3. 1.0% Agarose gel showing PCR-SSP products (194 bps in size) obtained using DNA isolated from 7 single-sperm cells captured from a slide smeared with a vaginal swab from couple #1. In some of the lanes is also visible an extra band with a lower molecular weight originated from primer-dimers. Size was confirmed using molecular DNA ladder (L). In parallel with the isolated single-sperm cells, a PCR positive control (+) and two negative controls (−) and (−−) were used.

the voluntary couples. Due to the low amount of mtDNA present in a single-cell, were performed two rounds of amplification with SSP. As previously shown, the high number of amplification cycles in this PCR approach makes the procedure more vulnerable to errors introduced by the Taq DNA Polymerase when compared with single step PCR protocols [23,24]. Examples of possible errors are false point mutations which can arise in an amplicon and be misinterpreted as a SNP after sequencing. In order to identify and exclude the errors produced during PCR, the SNPs obtained from the analysis of the sperm cell were compared with the SNPs obtained from the analysis of the buccal swab for the same individual. Additionally, as sequencing reactions are also based on DNA amplification and therefore able to generate errors, all products were sequenced in both forward and reverse directions to avoid mistakes in the attribution of SNPs. One other problem of two-rounds PCR strategies is related with the additional handling of free amplicons which increases the risk of generalised contaminations. In the present work extremely care was taken to reduce this risk. Different aliquots of all reagents were used for the preparation of the first and second round PCRs and the working stations were exhaustively cleaned with appropriate decontaminating solutions. Always as possible, the preparation of the two reactions was done in different laboratorial areas. In addition to these measures, the risk of contamination was also minimised by using SSP based on SNP unique for each couple and also different from those of the technician. For each of the 5 couples analysed the designed SSP allowed always to obtain a PCR product suitable to be sequenced. The complete strategy enabled the obtaining of clear sequences and mt-haplotypes from a single sperm cell isolated from the vaginal swabs. These mt-haplotypes matched completely (and exclusively) the mt-haplotype of the male. The lack of contamination with female genetic material was verified by electrophoretic analysis.

In forensic studies based on mtDNA analysis, the low discrimination power it is often pointed as a problem. A battery of mtSNP markers spread along the coding region of mtDNA can increase the comparatively low discrimination power of mtDNA studies [25]. Usually these SNPs located in the coding region are analysed with the aid of the snapshot analysis but theoretically, the SSP strategy here described could also be applied to their study. The approach here described for the control region of mtDNA can eventually be extended to mtSNP markers located in any place of the mitochondrial genome.

Once female cells do not have Y chromosome, the Y-STR analysis could eventually be considered as an alternative method. The analysis could be done without a prior knowledge of the suspect and so results would be more suitable to identify suspects by inquiring databases. These are two apparent advantages from the method when comparing with the mtDNA analysis described on the present work which is dependent on a previous mtDNA analysis of the suspect. But considering the biological material that was used on the present work (vaginal swabs) unfortunately the Y-STR method seems to have strong limitations: (i) Sperm cells present in vaginal swabs contain only one copy of nuclear DNA
(haploid cells) and it is often degraded. On PCR reactions where extremely low amounts of template DNA is present or where DNA is already partially degraded, Taq DNA Polymerase used for DNA amplification often produces false alleles or totally fails to amplify the STR-loci [9]. Given that the copy number of mtDNA templates is higher when compared with nuclear DNA templates and given that mtDNA is eventually more resistant to degradation by exonucleases [11], the probability of finding intact mtDNA templates on a partially degraded limited DNA sample is therefore higher. This might contribute decisively to avoid/diminish the above mentioned problems associated with the Taq DNA Polymerase. (ii) Y-STR method is not able to provide individual profiles from a mixture of genetic material belonging to more than one individual. In cases where more than one rapist is expected, it is necessary to discriminate the sperm cells belonging to each perpetrator. That can only be made by isolating single-cells and Y-STR fails to provide a good and complete profile of a single sperm cell. Moreover, from the sperm cells isolated from the vaginal swab, only 50% have the Y-chromosome while all of them carry several copies of mtDNA.

Exploring the possibility to test the same sperm cell for several suspects we altered the amplification protocol for one of the 5 couples tested (couple #5). Here, universal primers were used in the first amplification step allowing a non-specific amplification of all types of DNA present in samples. However, at this stage ampiclon and Y-STR were used only in the second round of PCR. Using this approach, the material of the first amplification would be sufficient for 8 parallel individual-specific analyses. Although this strategy requires more experimental verification the preliminary results are encouraging and it seems to be possible that even the genetic material of a single-cell allows the simultaneous identification analysis for several suspects of a sexual assault crime.

As documented in a previous study, the distinct separation of sperm cells from contaminating female cells exhibits a clear analytical advantage over the usage of non-purified material [2]. However, for the strategy described in the present work the separation requires a correct visual identification of the cells under a microscope. That procedure might be seriously compromised if sample is collected later than 72 h after intercourse as sperm cells tend to decompose and to lose their tails in the vaginal environment during this period of time [26]. Nevertheless, when the separation is no longer possible, the application of SSP-approach suggested in the present work to DNA samples collected from a mixture of cells might be worthwhile. As in most cases STR-approaches fail if performed after that time [9], specific amplification of mtDNA might be a successful alternative according to the arguments mentioned above.

Summarizing the strategy that we proposed in this manuscript and transporting it into a more practical dimension, in a first moment, the profile(s) of the suspect(s) and the victim throughout the control region of the mtDNA has to be obtained from standard biological material (e.g. buccal swabs) and with universal primers. SSP(s) would be designed for each suspect based on SNPs existent between the victim’s and suspect’s (and preferentially also the laboratory technician’s) profile. The SSP(s) should be chosen in order to allow studying the biggest part of the control region as possible. Optimization of the PCR conditions and assessment of the efficiency and the specificity of the designed SSP(s), should be done using dilutions of DNA obtained from reference samples (pure and in a mixture). In cases where samples are highly degraded, higher amplification efficiencies can eventually be achieved reducing the size of the amplicons and making a fractioned analysis of the whole region using more than one primer pair. Also one should consider the design of two SSP(s) (one reverse other forward) anchored on a same SNP in order to obtain the sequence of the complete control region.

In a second moment, and using the optimal PCR conditions verified previously, SSP would be used to amplify DNA obtained from single sperm cells collected from the vaginal swab (case sample). If no amplification is obtained, it is assumed as a lack of evidence for the suspect’s inclusion and, if possible, the analysis should be made for other sperm cells in order to confirm that we are really in the presence of a non-match. In case of successful amplification (visualized as a band in an agarose gel), the sequence of the PCR products must be verified using both primers present on the PCR reaction (sequencing in both directions). If the sequence obtained does not match with the reference sequence of the suspect, evidences exist to exclude suspect (although both sequences share the specific SNP used for SSP designed). If a similarity is observed between case sample and suspect reference sample, the suspect cannot be excluded from being the perpetrator.

The previous knowledge of the mt-haplotype of the suspect might eventually be pointed as a problem as it can bias the interpretation of the results obtained. Indeed, this it is not a blind analysis. Even so, and considering the undoubting results obtained and also the fact that the other available methods are not applicable to the specific situation covered on this study, in our opinion, the method here described is a valuable tool. Considering the interpretation of the obtained results as evidences and consequent admissibility in court, in our opinion the strategy here proposed (eventually supported by data collected from the coding region of the mtDNA) can lead to results as reliable as the other standard mtDNA analysis in terms of supporting suspect’s exclusions.

5. Conclusion

Here we describe a new protocol for mtDNA typing of a single-sperm cell isolated from vaginal swabs. The strategy overcomes the problem of contamination with DNA from the victim that usually occurs using STR. This methodology seems to be advantageous when compared with other methods particularly because it has the potential to be used in cases of multiple rapists allowing testing for multiple suspects using DNA from one single cell.

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