On the effects of sampling, analysis and interpretation strategies for complex forensic DNA research with focus on sexual assault cases

Benschop, C.C.G.

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Chapter 7

General discussion

Corina C.G. Benschop
Titia Sijen
Thesis results and implementation in casework

Two outcomes from this thesis stand out as actual tools that can be implemented in casework. The first is the use of nylon flocked swabs for intimate (vaginal) samplings in sexual assault cases, and the second is the use of the $n/2$ consensus approach for robust and informative analysis of low template (LT) DNA profiles. Laboratory experiments (described in Chapter 1 and [1]) showed that nylon flocked swabs are more efficient than the standard cotton swabs for the microscopic analysis and forensic DNA typing of post-coital vaginal samples. Moreover, nylon flocked swabs showed no practical complications during a five-months pilot study on sexual assault casework analysis in The Netherlands. These nylon flocked swabs were combined with an in-tube desiccating agent and incorporated in the revised Sexual Assault Forensic Examination (SAFE) kit (Chapter 2 and [2]) that is in use in The Netherlands since December 2010. For the $n/2$ consensus approach a similar strategy was followed. First, it was established that the approach performed superior to other consensus strategies when analysing relatively simple LT DNA profiles (as described in Chapter 4 and [3]). Next, we confirmed that the success of this approach also applies to complex LT DNA profiles (Chapter 5 and [4]). Finally, the $n/2$ consensus strategy was used effectively in a pilot study during which reporting officers assessed mimicked cases involving complex LT DNA mixtures (Chapter 6). The $n/2$ consensus approach has been implemented at the NFI since September 2010. Alleles detected in at least two out of three or four replicates are included in the consensus DNA profile. Dutch NFI partners were informed on these improved and implemented procedures by various publications [5-7].

Forensic developments and source level questions

Both the above mentioned achievements relate to the analysis of human biological evidentiary traces at source level. They aim to address the question “**Which individual did the biological material originate from?**”; the nylon flocked swabs are more efficient in sample collection and elution of intact cell material, and the consensus strategy enables more accurate data interpretation. One can distinguish four steps in the forensic analysis process where the efficacy of donor inference can be increased: (1) use of effective tools for the collection of cellular material, (2) application of efficient methods for the extraction of nucleic acids, (3) use of informative markers combined in efficient multiplexes and (4) application of effective interpretation strategies. All these steps are subject to research within the forensic community and are explored by manufacturers of products for forensic laboratories. Thus, progress is to be noted at various levels, which is described in the subsequent paragraphs.
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The range of sample collection items has been expanded with for instance, tape lifts to collect cellular material from clothing or skin [8], mini-tip swabs which allow sampling on small or grooved surfaces and swabs of alternative materials other than cotton, such as the above described nylon flocked swabs [1]. It is essential that these items are free of human DNA, and for now ethylene oxide treatment appears to be the preferred method [9,10]. DNA contamination during the production process can have large impacts on police investigations. This is evident from the ‘Phantom of Heilbronn’ incident where 40 crime scenes including six murder scenes in the period from 1993 to 2009 were linked by DNA profiling techniques to an unknown female [11]. In 2009 it was found that the generated DNA profile matched the reference DNA profile of an employee at the swab factory, where the swabs were being produced. These swabs were sterile, but not certified for human DNA collection and subsequent DNA profiling. Besides the developments regarding sampling items, specialised techniques relating to the collection of cellular material have been introduced to the forensic field. An advanced technique is laser microdissection (LMD), which can be applied to isolate specific cells of special interest from microscopic slides. In forensics, LMD is used to capture spermatozoa or male diploid cells amongst a surplus of female (vaginal) cells for which the male diploid cells need to be marked by XY-fluorescence in situ hybridisation (XY-FISH) labelling (e.g. [12,13]). LMD can also specifically collect the regions of maternal or foetal origin from aborted foetuses or umbilical cord material. Again, the cells of interest need to initially be selected by histological staining or other labelling techniques. Furthermore, it was recently shown that the individual collection and analysis of skin flakes has the potential to unravel the contributions of multiple donors in contact traces [14]. The DNA content of skin flakes can vary greatly both between and within donors, and many flakes do not yield a DNA result. Therefore, this rather elaborate method seems only applicable to a limited number of cases.

For the efficient extraction of pure DNA, silica-based methods are most prominent. DNA molecules bind to the silica surfaces in the presence of certain salts and under specified pH conditions. A wash step then attempts to rid the solution of any agents that may inhibit the DNA amplification step. Silica can have various formats like columns, beads or magnetic beads. An additional advantage is that silica carriers can be combined with both automated DNA purification robotic platforms and with microfluidics. The latter forms the basis of mobile DNA analysis technology [15]. The goal of mobile technology is to enable DNA analysis at the scene of crime in order to obtain early investigative leads. The release of several mobile platforms is expected for 2012. An additional development, which resulted in commercial products from 2009 and onwards, is to proceed to short tandem repeat (STR) amplification without a DNA extraction step, thus reducing time and costs in obtaining DNA results. Single source reference samples particularly befit these direct STR profiling kits (like AmpFISTR® Identifiler® Direct and PowerPlex® 18D), and clear instructions regarding
the size of the punches of blood samples deposited on collection-cards accompany the kits. This is essential for obtaining complete and balanced STR profiles. At the NFI, an in-house developed DNA-6-hours service based on rapid and direct PCR was launched in October 2010 [16,17]. This service aims to rapidly derive DNA profiles from various types of crime scene traces to assist the police early on in their investigations. The method includes tape-lift collection of deposited cellular material, taking only a limited amount of the trace, while the remainder stays dry and intact for analysis using standard procedures.

In the past years, the number of markers in autosomal STR typing kits has increased from 3 to 15 or 16 (plus the gender-typing marker amelogenin). The kits released around the year 2010 included not only a higher number of markers, but also markers with a higher discriminative power [18,19], such as SE33, which is the most discriminative forensic marker. Furthermore, these new STR kits included markers of small amplicon size (mini-STRs, which have primer binding sites close to the repeat region), with profiles starting from 70 bases instead of 100. Reduced size amplicons assist in the amplification of degraded DNA [20]. Single nucleotide polymorphisms (SNPs) have been described as an alternative strategy to analyse severely degraded DNA samples. Both bi-allelic and tri-allelic variants [21,22] can serve forensic purposes, but SNP typing results can only be used for direct comparisons to reference SNP profiles as the DNA databases only contain STR data. Next to these autosomal markers, Y-chromosomal, X-chromosomal and mitochondrial DNA (mtDNA) markers have been developed for forensic use [23-26]. For the Y-chromosome the most applied forensic markers are again STRs (although Y-SNPs are also described). Y-chromosome analysis is mostly applied when a surplus of female DNA is present and the male component cannot be deconvoluted in the mixed autosomal profile. Y-STR profiles result in information regarding the paternal lineage, although close paternal relatives can most often not be discriminated (only the recently described rapidly mutating Y-STRs appear able to distinguish close relatives [24]). X-STR analysis has the potential for kinship cases where a daughter-father relationship is in question (since a daughter inherits the X-chromosome the father has). mtDNA analysis is mostly applied to hairs or skeletal remains, as for these samples nuclear DNA is often lacking or degraded. The introduction of mtDNA multiplexes of reduced size amplicons has increased the success rate of mtDNA sequencing for highly degraded samples. Due to the high number of mtDNA copies in a cell, mtDNA analysis is highly sensitive. However, it is maternally inherited and therefore only information regarding the maternal lineage is obtained. mtDNA sequencing is foremost based on Sanger sequencing protocols (chain termination). Many new sequencing procedures have been developed, such as the next generation sequencing procedures that are mostly based on emulsion PCRs, and the third generation sequencing strategies that predominantly rely on nanopore technology and real-time monitoring. These methods extend forensic sequencing from
single read sequencing of amplified fragments (STRs, SNPs, mtDNA) to deep coverage
sequencing (many reads per amplicon) and even whole genome sequencing by directly
reading the DNA. Researchers will explore these technologies, the forensic applications
and the legal aspects, and without doubt exciting developments lay ahead.

Interpretation of the DNA profiling results includes, among others, the determination
of the reliability of the results: meaning for instance, with STR-typing, inferring whether
the DNA profile is stochastically affected (e.g. are alleles missing) and discerning true
allele calls from artefact peaks. These issues are particularly prevalent with LT DNA
profiles, which usually contain stochastic amplification artefacts as described in Chapters
4, 5 and 6. In addition, to weigh the evidential value, the rarity of the DNA profile needs
to be established. The allele frequencies in a relevant reference database are used
to evaluate this aspect. When new markers are included in the standard STR-typing
set, these population databases need to be updated. The use of more discriminative
markers requires that larger datasets be analysed to obtain reliable frequencies for
the less common alleles. After DNA profile interpretation, profiles are compared to
the reference profiles of persons of interest, and the evidential value of the result of
this comparison is weighed. With a match between the DNA profile of a reference
sample and a high template trace sample, the rarity of a DNA profile determines the
match probability. For complex samples (e.g. having multiple contributors, low template
components, contributions of related donors), it is generally more challenging to weigh
the evidence (e.g. [27-30]). Probabilistic models hold promise for the (near) future,
although non-probabilistic approaches (e.g. [31]) have value as well and may provide
useful tools for the mean time.

When a case lacks a suspect(s) whose reference DNA profile(s) can be compared
to the trace profile, and no possible matches are obtained upon searching DNA
the databases, one may need to proceed to other approaches. One can examine
“Whether a close relative of the perpetrator is present in the DNA database?” and
apply familial searching. Specifically developed software may be used to generate a
list of those offenders in the DNA database that are most likely to be a close relative
of the perpetrator. Extensive autosomal typing, Y-chromosome profiling and possibly
even mtDNA analysis can disprove or confirm familial relationships and reduce the
length of the list. Next, police investigations are required. Final confirmation can be
obtained when a suspect gives a sample for DNA profiling and suspect and traces
profiles are compared. An alternative to familial searching is to assess “What is the
phenotypic appearance, age and ancestry of a donor?”. During the last years progress
has been made regarding the prediction of externally visible characteristics such as
eye, hair and skin colour. To this aim, specific SNPs located in and nearby genes that
have a role in pigmentation are analysed (e.g. [32-36]). To allow application to forensic
cases, much effort was taken to keep the number of SNPs as low as possible so
that a single, sensitive SNaPshot multiplex would suffice to address phenotypic traits.
For some cases it is relevant to assess the ethnic origin of an unknown perpetrator. Information regarding the geographical origin lies enclosed in both autosomes, the Y-chromosome and mtDNA, and many SNP markers have been described that assist biogeographic ancestry inference [37-41]. Evidently, ethnic origin inference becomes more complex when ethnic admixture occurs, an event that is very common in current world populations. Another relevant phenotypic trait is age. Although several molecular changes occur when a human being ages (such as telomere shortening and the accumulation of deletions in mtDNA), the development of DNA markers that may have forensic potential has remained difficult. Recently, a sjTREC assay was reported that is based on the detection of a circular DNA molecule that results from T-cell DNA rearrangements in the thymus. As the thymus involutes with age, the number of sjTREC molecules can predict the age of the donor in a bloodstain. The standard error is ± 8.9 years [42], which is a too wide range for forensic casework, and additional research is still much needed in this area.

Forensic developments and activity level questions

In addition to the source level, two other levels can be addressed in forensic casework, namely activity level and offence level [43,44]. The offence level (“Did the suspect commit the offence?”) can be seen as the ultimate matter to be proved or disproved, and is clearly outside the domain of the forensic scientist. Forensic scientists do in some cases survey the activity level (“What activity led to the deposition of the cellular material?”). This question has several aspects, and some will be addressed below.

The question “Where does the cellular material related to the crime reside at the crime scene?” is a crucial first step for forensic analysis. Trace recovery is based on hypotheses regarding the course of events, criminalistic insight and also technical methods that aid in revealing the presence of (human) cellular material. Developments regarding the visualisation and the detection of biological material encompasses alternate light sources, sprays and spectral cameras [45-48]. These methods aim for the detection of biological material in an easy, fast, and if possible non-invasive and/or non-destructive manner.

The predominant question assessed when analysing the activity level is “What is the origin of the cellular material in the crime scene trace?” To answer this question, the following five strategies can be applied. The first is the microscopic analysis of slides prepared from the evidentiary item. Since specific cell types are detected from a characteristic morphology or staining pattern, microscopic analysis requires that cells of recognisable morphology be eluted from swabs or textiles. Spermatozoa are the prevalent cell type that is searched for using forensic microscopy. The second approach
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involves presumptive or confirmatory tests. These are generally based on the presence (or relative high abundance) of certain proteins or enzymes in specific body fluids, and are detected by antibodies or responsive reagents. Presumptive tests are performed prior to DNA extraction (proteins become degraded during the extraction process) and exist for blood, seminal fluid, saliva, and urine [49]. The third approach involves RNA cell typing [50-52], which assesses the presence of body fluid-specific (m)RNAs. RNA molecules are generally less stable than DNA molecules due to their single-stranded nature. However, when small amplicon sizes are used, RNA analysis of forensic stains is possible. With RNA cell typing the presence of blood, semen, saliva, vaginal mucosa, menstrual secretion and skin can be indicated. RNA cell typing requires that not only DNA but also RNA is isolated from a forensic stain, and does therefore not apply to stored DNA extracts (personal communication Lindenbergh and coworkers). Recently, a fourth approach was reported to hold potential for cell type analysis and interestingly, this method may be compatible with existing DNA extracts as it assesses the level of DNA methylation [53,54]. DNA methylation is a crucial part of cellular differentiation and development in higher organisms. It typically occurs in a CpG dinucleotide context. Specific CpGs were found to be differentially methylated in various tissues and especially for semen proof of concept was shown, as this body fluid could be distinguished from other cell types by DNA methylation markers [53,54]. DNA methylation can be assessed by the use of methylation sensitive restriction enzymes (when corresponding restriction sites reside around the differentially methylated positions) or by bi-sulphite sequencing techniques, which have the drawback that relatively large amounts of DNA are needed. Third generation sequencing approaches have been described that assess not only the DNA sequence, but also the base modifications [55]. Such techniques may hold great promises for the future of forensic biological research. A fifth approach relates to the use of microbial flora to indicate a body site origin (as described in Chapter 3 for vaginal origin) [56,57,58,59]. This approach may be especially interesting to discriminate between biologically similar tissues at different body locations such as the vaginal and buccal mucous membranes; with mRNA profiling vaginal and buccal epithelia are rather complex to distinguish, while the microbial communities that inhabit the vagina or the mouth may differ substantially. Microbes can be detected by DNA (Chapter 3 and [59]) or RNA sequences [56]. Notwithstanding the various methods that are available to assess the cell types in a stain, serious complications occur with mixed and compromised forensic samples.

Another aspect that can assist in activity level research is answering the questions: “What is the deposition age of a trace?” and “At what time of the day was the trace deposited?”. These questions aim to increase insight in the course of events at the scene of crime. Also, they may inform on the relevance of a trace at a crime scene. So far, scientists are able to estimate the age of a blood stain with an uncertainty margin of 14 days (if environmental conditions are stable) using reflectance spectroscopy [60]. The
method measures hemoglobin and hemoglobin derivates, such as met-hemoglobin and hemichrome, via this non-invasive spectral imaging approach. To distinguish between traces deposited at late night and early morning (assuming a normal biological rhythm), circadian biomarkers are used [61]. Daylight is the predominant external factor that stimulates the endogenous circadian rhythms. In the proof of concept study, two circadian hormones, melatonin and cortisol, were analysed. Future studies will surely bring more details to such circadian analyses.

**Concluding remarks**

The broad range of developments described above illustrate how forensic researchers put efforts into improving methodologies, techniques and expertise relating to forensic science, and how they aim to answer an increasingly wide range of questions. Aspects that should not be ignored by forensic researchers are the less technical ones such as “What are the pitfalls when applying a technique to forensics?”, “How to interpret laboratory results?” and “What is the evidential value of these results?” To this aim, validation and implementation studies are essential. Furthermore, effort should be spent on explaining laboratory results and outcomes to for instance juridical professionals. Results from forensic laboratories are used in court, and can have large impacts on the lives of suspects, victims and their next of kin. Consequently, it is relevant to question, “What is the ethical and social impact of a new development in forensics?” and “Is additional legislation required?”. With that, it is important to translate impacts of laboratory results to politicians and the general public. Without social support forensic developments stand empty-handed.

**References**


