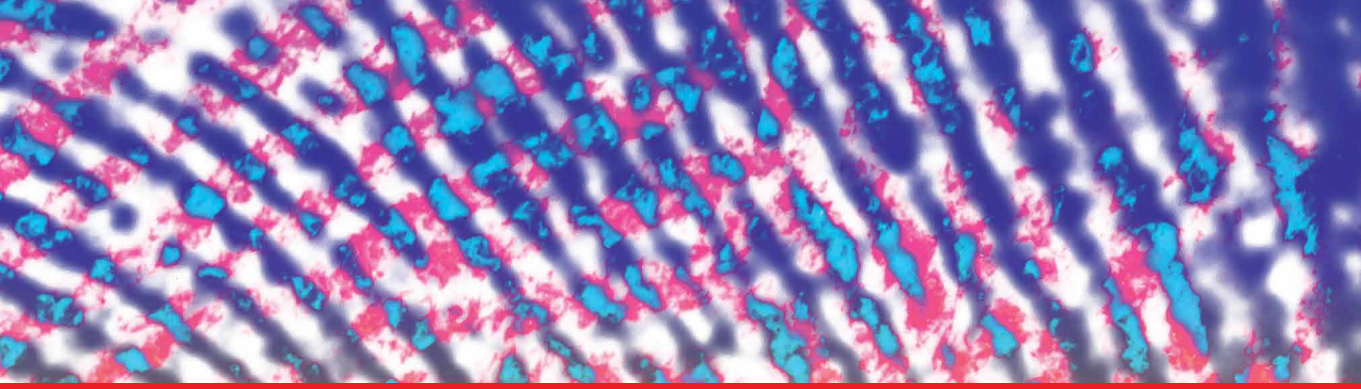

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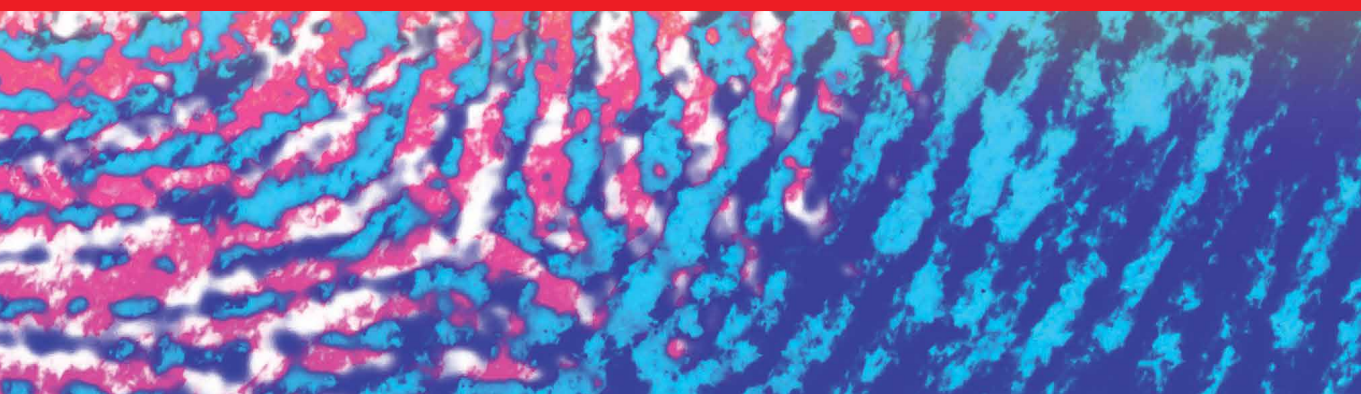
Forensic Analysis

Scientific and Medical Techniques and Evidence under the Microscope

Edited by Ian Freckelton



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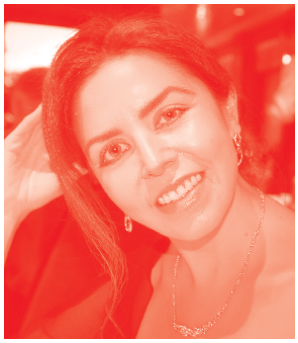

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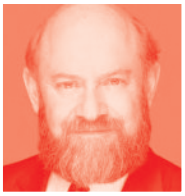
Meet the editor



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Contents

Preface

The Book

Forensic Analysis - Scientific and Medical Techniques and Evidence under the Microscope is an edited collection of cutting-edge analyses of diverse contemporary aspects of forensic science and forensic medicine. It is a truly international collection. It contains eleven chapters from scholars in Australia, China, Egypt, France, India, Iraq, Italy, the Netherlands, Russia and Saudi Arabia. The aim of the collection is to provide information about the parameters of expertise in relation to a number of areas that are being utilised as a part of criminal investigations and that are coming before courts internationally or will soon do so.

Reliability and Probative Value

Key aspects of the evaluation of forensic science and forensic medicine are the concepts of reliability and validity. While validity focuses upon the accuracy of a measure, in forensic matters reliability tends to be the more important issue in terms of the value of expert evidence. Evaluation of the reliability of expert opinion evidence is controversial in all countries and there are diverse yardsticks by which it can be measured, including formal recourse to factors such as falsifiability, error rate, extent of publication in recognised, peer-reviewed journals and reputation amongst colleagues in the same field. When areas are "novel" in the sense of still emerging from the iconoclastic or emerging toward the generally accepted by reference to objective criteria, there can be a tension between the actual techniques and the theories underlying them and the prejudicial effect that they may exert on courts (especially jurors) called upon to evaluate them. This can be so for countries that utilise admissibility criteria for such evidence but also for countries that focus upon the need to determine the probative value of such evidence.

In all such instances, there is a need for scientific and medical rigor with the employment of standardised methods, criteria for interpretation and retention of primary samples for re-testing as required. The caliber of those who undertake the forensic work is critical, as is the quality of the testing facilities, and adherence to ethical probity in disclosure and interpretation of results that may be other than criminal investigators are hoping for. There is always a need for transparency of processes and candid acknowledgment of the status of techniques and theories that as yet may not have fully fledged statistical bases enabling definitive interpretation of test results. In such scenarios courts and tribunals need to exercise circumspection in the reliance that they place upon such evidence, ensuring that witnesses giving evidence about such matters are made accountable for the opinions that they express. This highlights the need for legal practitioners to make themselves sufficiently familiar with the controversies about and limitations in scientific and medical techniques to be able to ask probing questions that expose parameters of expertise and accuracy.

The Chapters

The book begins with a review by Freckelton (Chapter 1) of the controversial and emerging area of **forensic gait analysis evidence**, which is given by practitioners from a number of disciplines, including podiatry, photogrammetry, biomechanics and anatomy, and has been the subject of diverse judicial decisions about admissibility in Canada, the United

Kingdom and Australia. The conclusion reached by the chapter is that the reception of forensic gait analysis evidence by the courts is dangerous and, as yet premature, until further development of statistically rigorous databases of gait characteristics.

Al-Dalmoshi et al. (Chapter 2) explain the ways in which **mitochondrial DNA** has the potential to be an important tool for human identification and differentiating between human and animal blood at crime scenes. In extreme conditions nuclear DNA is adversely affected or even destroyed, but mitochondrial DNA has characteristics that can be valuable for forensic purposes; amongst other things, it contains multiple copies and resists harsh and unstable conditions. It can be of particular utility in investigating blood sports and the provenance of blood samples found in diverse crime scenes.

Sahajpal et al. (Chapter 3) identify the threat to the environment and biodiversity from the commission of crimes involving wildlife. They note also the monetary values of the illegal trade in wildlife and therefore the temptation for the unscrupulous to engage in conduct that jeopardises the viability of many species. They review the three most frequently used techniques in **wildlife forensic investigation:** microscopy, DNA analysis and isotope analysis. They observe that some of the techniques employed in wildlife forensics require standardisation and that there are particular analytical challenges when evidentiary material is very limited.

Ioganson et al. (Chapter 4) review the reliability and reproducibility of **DNA profiling from degraded samples** by reference to extensive experience with analysis of bones and tissues in unidentified corpses in Russia.

Knoops (Chapter 5) describes the relatively new DNA technique of **probabilistic genotyping**, which has the potential to provide a determination of complex DNA profiles from multiple contributors. The author compares its utility to that of mainstream DNA techniques such as Combined Probability of Inclusion. The chapter highlights the potential pitfalls in traditional DNA test results where the samples are complex, as well as the importance of the information provided to the DNA tester. The chapter also emphasises the potential for probabilistic genotyping to correct wrongful convictions and to discern the real perpetrator of a crime where there are a number of suspects.

Kumar et al. (Chapter 6) demonstrate the many ways in which **tool mark analysis** can be utilised in criminal investigations. They review contemporary methods for forensic tool mark analysis and interpretation, noting that it can constitute an adjunct for contributions as diverse as anthropology, archaeology and pathology. They identify the need for forensic examination to be rational, unambiguous, balanced and rigorous.

Garg et al. (Chapter 7) review methods in **forensic osteology** for identifying deceased persons by reference to race, age and sex from bones. They argue that the discipline has emerged as an important means of identification within the criminal justice system and explain how certain bones can provide information about gender, race and other individualising characteristics relevant to criminal investigations.

Shetty and Shetty (Chapter 8) describe the field of **forensic diagnostics**, utilising biomarkers. They identify that pregnancy diagnosis from traces of blood can assist in finding a missing woman who is pregnant and in investigating illegal abortions. They also review the evidence for the utility of the technique for investigating sexual assaults, maternal substance abuse and paternity testing.

Scendoni (Chapter 9) reviews the literature on the forensic application of **saliva testing** as an alternative biological matrix to blood for the determination of xenobiotics and/or drugs of abuse and their metabolites in both the living and the dead. He highlights

recognised and potential forensic issues in the testing of saliva from a toxicological and medico-legal perspective, concluding that it requires highly qualified personnel who are able to apply analytical methods and interpret results in the light of up-to-date knowledge, and toxicological laboratories equipped with state-of-the-art instrumentation.

Aly et al. (Chapter 10) review the phenomenon of Tramadol-caused deaths and the potential for molecular autopsies to assist investigations into such drug-related deaths. They argue that **pharmacogenetic** and **toxicological investigations** can assist clinicians and courts alike by predicting and identifying adverse consequences from the administration of medications such as Tramadol.

Agrawal and Pandey (Chapter 11) conclude the collection, describing the emerging area of **forensic analysis of herbal medications**. They point out that patients are often unaware of the authenticity of drugs marketed as herbal, but that such medications may be adulterated by a range of products. This means that there is a significant public health need (especially in the time of COVID-19) to investigate the bioconstituents of such drugs. However, the authors acknowledge that forensic analysis of herbal medications is in its early stages and as yet its methodology needs to be standardised and validated.

Aspirations of the Book

Forensic Analysis - Scientific and Medical Techniques and Evidence under the Microscope is an innovative and contemporary international collaboration by scholars in medicine, science and law. It does not purport to be the last or the authoritative word on any of the diverse medical, scientific or legal issues that it canvasses. My hope as its editor is that it raises new issues, provides helpful information about emerging and conventional areas of forensic medico-scientific investigation, and gives a fillip to the development of rigorous international standards so that expert opinion evidence utilised by criminal investigators and relied upon by the courts will be both reliable and valid. In these circumstances, miscarriages of justice will be minimised, the respect of the community for the legal system will be vindicated, and the integrity of the many disciplines of forensic science and medicine will be enhanced.

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Adjunct Professor,
Johns Hopkins University,
United States

Dedication

For Otis and Penny,

The Best of Pandemic Companions

Section 1

Novel Scientific Evidence

Chapter 1

Guarding the Gait: Evaluating Forensic Gait Analysis Evidence

Ian Freckelton

Abstract

Novel scientific evidence challenges courts in terms of how they can evaluate reliability for the purposes of making admissibility decisions and assigning probative value to information that is adduced before them. An example of such problematic evidence is forensic gait analysis evidence which is in its infancy as a discipline of forensic science. This chapter reviews how objections to forensic gait analysis evidence have been handled in judicial decisions at first instance and on appeal in Canada, the United Kingdom and Australia. It identifies vulnerabilities in such evidence, especially when jurors are required to incorporate expert opinions (often from podiatrists) about the similarities in gait between that of the accused and a person seen on CCTV footage. The chapter expresses concern about the current scientific basis for such evidence in the absence of well developed databases in relation to gait characteristics, difficulties that characterise interpretation of CCTV footage, and the role that subjective issues can play in analyses by experts in gait interpretation. It notes a United Kingdom initiative in formulating a code of practice for forensic gait analysts but calls for caution in relation to reception and weight to be attached to such evidence until its scientific status becomes more developed.

Keywords: Novel scientific evidence, forensic gait analysis evidence, admissibility, probative value, weight, miscarriages of justice

1. Introduction

Forensic gait analysis is one of a number of novel areas of scientific evidence that have troubled the courts in recent years and led to conflicting curial decisions in relation to admissibility and probative value. In this regard it takes its place alongside facial mapping and body mapping evidence, psychological autopsy evidence, polygraph evidence, lip-reading evidence and ear print comparison evidence [1] as a form of evidence that carries the potential to appear to be more scientifically reliable than it is and therefore which carries a risk of misevaluation if not critically scrutinised. Such evidence is particularly problematic in criminal trials when verdicts are determined by lay jurors. This is because of the danger that decision-makers will invest overmuch trust in evidence that appears to be scientifically rigorous when it may not be [2].

Forensic gait analysis functions as an example of a dangerous category of apparently scientific evidence that is beguilingly probative but which has the potential to lead to miscarriages of justice. It challenges decision-making by courts and attempts by law reformers to formulate criteria for determining admissibility or at least indicia for safeguards so that misimpressions are not given about evidentiary reliability

which may lead to miscarriages of justice. Further, it raises questions about those who are prepared to hold themselves out as forensic experts in the area.

This chapter reviews the current status of knowledge about forensic gait analysis. It identifies significant judicial decisions about the area and it reflects on measures that need to be adopted to minimise the risks that inappropriate reliance will be placed upon problematic application of this form of evidence in criminal trials before it is developed further and acquires the attributes of scientific reliability.

2. Forensic gait analysis

Gait is the way in which a person takes steps when walking or running. It consists of a series of largely repetitive, cyclical movements that are generally symmetrical. It can be observed at a distance, without the knowledge of a person of interest and cannot easily be concealed or dissimulated. Gait analysis has been defined as 'the systematic study of human walking, using the eye and brain of experienced observers, augmented by instrumentation for measuring body movements, body mechanics, and the activity of the muscles' [3–5]. It can be undertaken by observations that a person is bow-legged or has knock-knees or a has a foot that points in or out. It can include consideration of the joints and segments of various parts of the body, including the hips. A variety of tools can be employed to enable calculation of lengths or angles.

Forensic gait analysis is the analysis of the style or manner in which a person walks, as applied to legal issues, including in the context of criminal trials. It has emerged as an important aspect of forensic podiatry, which has been defined as 'the application of sound and researched podiatric knowledge and experience in forensic investigations to show the association of an individual with a scene of crime, or to answer any other legal question concerned with the foot or footwear that requires knowledge of the functioning foot' [6–8]. The area of forensic gait analysis is an evolving and controversial area of scientific controversy [5, 9–12] that generally involves the use of closed circuit television ('CCTV') footage that can be of variable quality in which a comparison is made of a criminal perpetrator's gait with that of the accused. A number of judicial decisions on the issue of admissibility have been handed down, including on appeal in Canada (*R v Aitken*) [13, 14]; the United Kingdom (*Otway v The Queen*) [4], (*Hashi v The Queen*) [15] and Australia (*R v Crupi*) [16].

3. Canadian authority

In *R v Aitken* [13] the accused was charged with murder. There were no witnesses but the fatal shooting outside a Chelsea apartment was captured on the security CCTV positioned above the doorway to a building. Eighteen hours before the shooting CCTV also captured a person of similar size and stature as the shooter apparently canvassing the area and inspecting the buzzer system (the person of interest). The prosecution proposed to call persons whom it proffered as experts from two different but, it maintained, complementary disciplines.

The first expert was Hayden Kelly who held a degree in podiatric medicine and had been practising since 1987 in the United Kingdom. He had seen approximately 38,000 patients. In 2002 he became a podiatric surgeon. From 2006 to the time of the trial in 2008 he had been a researcher in forensic gait analysis identification at Barts College, which is part of the University of London. He had been consulted in approximately 150 legal cases and written over 50 expert opinions. He had testified in the English criminal courts over twenty times.

The second expert was Dr. Vernon Brugge who held a doctorate in biology and was held out by the prosecution as an expert in examining photographic evidence. He joined the Federal Bureau of Investigation as a forensic scientist who examined photographic evidence in the technological digital evidence laboratory in Virginia. He had given expert evidence in forensic image analysis, including photographic comparisons, photogrammetry, image and video authentication on some two dozen occasions. He had performed facial and clothing comparisons hundreds to thousands of times. Dr. Brugge's evidence included photogrammetric evidence and facial and clothing comparisons. In his work undertaking photographic comparison, Dr. Brugge compared features on an object or person depicted in a questioned image with a known image to identify or exclude from similarity, utilising 'class characteristics' -those that are common to a group or class of people - and 'individual characteristics' identifying characteristics that separate someone from the class.

Mr. Kelly described his forensic gait analysis procedure as having three steps:

1. He views video footage (or stills) of an individual to determine:

   a. If the quality of the film is sufficiently clear, and

   b. If there is a particularly distinctive gait or feature of gait shown.

2. If he finds distinctive features, then he views other footage of an individual(s) to see if the same features or gait exist;

3. Following that, the analysis is performed on a control, which is footage of a known individual(s) ([13] at [10]).

His view was that the person of interest, the shooter, and the accused shared a "very strong likeness" in that they all had 'very abducted left and right feet and an everted left foot' ([13] at [13]). He expressed the view that he 'would not expect to see this in more than one percent of the population. He did not observe anything in the material with which he was provided that suggested that the accused could not have been the person of interest or the shooter depicted in the videos ([13] at [13]). Thus his testimony amounted to identification of points of similarity and no points of dissimilarity.

Dr. Brugge used Photoshop techniques and a power point program to enhance and overlay known images on enhanced questioned images to detect similarities and differences. He compared still photographs from the video outside the Chelsea apartment where the murder took place with photographs of the accused. His evidence was he could not identify the accused as the person of interest or the shooter, but he could not exclude him either. Dr. Brugge could find no 'individualizers', such as scars, marks, moles, etc., that could lead to positive identification or exclusion of any of the individuals depicted in the photographs. Similarly, the clothing worn by the person of interest or the shooter bore no individual identifiers such as rips, tears or stains. The defence accepted that Dr. Brugge could testify as to how the video and video stills were processed to improve details by adjusting brightness and contrast and image enhancement but not the photographic comparisons.

Justice Satanove applied the Canadian tests in relation to expert evidence admissibility set out by the Supreme Court in *R v Mohan* [17], requiring establishment of:

1. Relevance;

2. Necessity in assisting the trier of fact;

3. The absence of any exclusionary rule; and.

4. A properly qualified expert.

She found the evidence of Mr. Kelly to have 'high probative value' in that it tended to establish a fact in issue, the identity of the killer ([13] at [19]). However, she concluded that Dr. Brugge's evidence was not admissible although she permitted his evidence on the technological enhancements he made to the videos and stills that were shown to the jury.

Justice Satanove accepted that: 'Evidence that is otherwise logically relevant may be excluded if its probative value is overborne by its prejudicial effect; if it involves an inordinate amount of time that is not commensurate with its value; or if it is misleading in the sense that its effect on the trier of facts, particularly a jury, is out of proportion to its reliability. There is a danger that expert evidence will be misused and will distort the fact-finding process. Dressed up in scientific language that the jury does not easily understand, and submitted through a witness of impressive credentials, the evidence may lie be accepted by the jury as being virtually infallible and, thus, given more weight than it deserves' ([13] at [19]). She observed that the potentially prejudicial effect of the experts' evidence may in their impressive credentials and their aura of infallibility ([13] at [19]).

Tellingly, she noted that Mr. Kelly drew a conclusion as to the degree of prevalence of gait characteristics in the general population 'that appears significant, yet lacks a scientific basis' ([13] at [23]). To address this risk, she allowed Mr. Kelly's evidence but not his opinion about the extent of abduction and eversion in the general population. However, Justice Satanove was not satisfied that Dr. Brugge's evidence was 'necessary', as that term is understood under Canadian law, namely that such evidence is outside the experience and knowledge of a judge or jury. She accepted that it may be '"helpful" as an experienced person's observation of facial features and clothing, but the jury will be able to form their own conclusions without this help. In my opinion, Dr. Vorder Brugge's comparison evidence does not offer any information outside of the purview of the ordinary jury, and therefore does not meet the test of necessity' ([13] at [24]).

She was not satisfied that the evidence of Mr. Kelly constituted "novel science": "podiatry has been in existence for a thousand years and the expertise of a podiatrist to analyze an individual's gait has long been accepted and practiced in a clinical setting. After carefully viewing the video frame by frame, many, many times, with his trained and practiced eye, he is able to point out fairly unique characteristics of the gait and stance of the individuals depicted in the video. The features are akin to individual identifiers to some extent. I do not think there is a danger of the jury being mesmerized by what is quite simply an exercise of expertise in observation and diagnosis" ([13] at [34]). The result was that Justice Satanove admitted the gait analysis evidence of Mr. Kelly, save the part which referred to a mathematically expressed degree of prevalence but excluded the evidence of Dr. Brugge insofar as it related to technological enhancements he made to videos and stills, but excluded his comparison evidence.

Cunliffe and Edmond [10] have criticised the ruling by Justice Satanove as an 'admissibility compromise. However, on appeal (*R v Aitken* [14]) the British Columbia Court of Appeal [14] upheld the decision at first instance to admit the evidence of Dr. Kelly. The Court applied the *Mohan* test formulated by the court in *R v Abbey* [18] at [80] where it was held that a trial judge must conduct a 'rule-based' analysis to assess compliance with preconditions to admissibility:

- the proposed opinion must relate to a subject matter that is properly the subject of expert opinion evidence;

- the witness must be qualified to give the opinion;
- the proposed opinion must not run afoul of any exclusionary rule apart entirely from the expert opinion rule; and
- the proposed opinion must be logically relevant to a material issue.

The Court of Appeal agreed with Justice Satanove that the evidence of Mr. Kelly was not 'novel' for the purposes of the *Mohan* test and concluded that his evidence fell into the category of 'specialized knowledge gained through experience and specialized training' ([14] at [80]). It held that this meant that there was no error in the trial judge failing to consider indicia of scientific validity such as peer review, rate of error and adherence to a scientific method – 'These factors have limited relevance in a case like the one at hand where a witness's expertise is gained over a period of years through observation and experience in the professional realm' ([14] at [80]). The Court of Appeal concluded that Mr. Kelly's evidence that there was a 'very strong likeness' in gait between the individuals shown on the video footage was 'merely a distillation of his factual observations, and that he did not usurp the role of the trier of fact by offering a conclusion on the ultimate issue of the identity of the shooter' ([14] at [84]). This meant that he was not breaching the preclusion on giving evidence on an ultimate issue but simply offering a piece of circumstantial evidence that narrowed the gap between who could be the shooter and the person of interest and the accused.

In important respects, the most contentious aspect of the Court of Appeal's judgement was its endorsement in the context of forensic gait analysis ([14] at [85]) of the comments of Justice of Appeal Doherty in *Abbey* ([18] at [92]):

> *… I doubt that the jury would have difficulty critically evaluating [the expert's] opinion. There was nothing complex or obscure about his methodology, the material he relied on in forming his opinion or the language in which he framed and explained his opinion. As when measuring the benefits flowing from the admission of expert evidence, the trial judge as "gatekeeper" must go beyond truisms about the risks inherent in expert evidence and come to grips with those risks as they apply to the particular circumstances of the individual case.*

The Court of Appeal found no error in the evidence of Mr. Kelly being placed before the jury to provide assistance in their evaluation of the images so that the jurors might utilise the knowledge acquired by Mr. Kelly through his training and experience in arriving at their own conclusions regarding the identity of the shooter ([14] at [86]). The Court of Appeal reviewed English decisions on the issue, including that of *Otway v The Queen* [6] (see below), which had been decided since the trial judge's decision, and classified them to be of persuasive authority and supportive of the admissibility of forensic gait analysis in the circumstances of what was before the Court.

While Cunliffe and Edmond [10] have been critical of the latitude extended by the trial and appellate decisions as to the admissibility of forensic gait analysis in the *Aitken* judgements, it is apparent that at both levels the courts regarded the evidence as having probative value which the jury was in an adequate position to evaluate. Therein lies the contentious and problematic aspects of the judgements in that the jurors had no statistical tool on the basis of which to evaluate the significance of the points of similarity identified by the expert and there was a risk of their attributing weight and giving deference to the evidence which was out of proportion to its probative value.

4. English authority

Although gait analysis evidence was permitted in the 1839 case of *R v Thomas Jackson* where the accused was identified by his bowed left leg and his propensity to walk with a limp, and was admitted in an armed robbery trial in the Old Bailey in 2000 in relation to a suspect said by a podiatrist to have a bow-legged gait encountered in only 5% of the United Kingdom population, (*R v Saunders*: see [5]), there are two major English appellate decisions in relation to gait analysis.

4.1 R v Otway

In *Otway v The Queen* [6], evidence in relation to walking gait analysis was adduced in a murder trial by the prosecution from CCTV images which depicted 20 seconds of an offender in motion. The driver and a passenger of a motor vehicle approached the deceased man and the passenger shot him dead. Otway was alleged by the prosecution to be the driver and was convicted on the basis of being participant in a joint homicidal enterprise.

The witness who gave the gait analysis evidence was a Mr. David Blake, who described himself as a 'podiatrist and specialist in lower limb gait, pathomechanics and biomechanics' ([6] at [10]). He said that he routinely used video camera equipment to analyse gait clinically to assess and diagnose anatomical and skeletal conditions. He described the practice of gait analysis ([6] at [10]) as follows:

> *Gait analysis is the examination of walking or running. The gait, or walking cycle, skeletal movement in general can have recognised anatomical movements or reference points during a walking cycle. Biomechanics is the examination and analysis of body movement. Specifically in this case to humans the skeleton can at times give an anatomical signature that if not unique, can be a relatively rare anatomical position or movement to a few individuals. A person's walking cycle or his skeletal anatomy is difficult to hide as it is part of their body's anatomy. The science of gait analysis was introduced into the UK and the profession of podiatry in the early 1970s. The gait cycle can be broken down into factors such as the position of feet and other parts of the lower limb. Thus features of gait can be identified and sometimes quantified. Podiatrists use gait analysis virtually every day in their practice. Recently that science has been applied forensically. The Council for the Registration of Forensic Practitioners recognises gait analysis and footprint identification as important components in identification of individuals. A podiatric section has recently been set up ... some clinicians may suggest that certain key elements of gait cycle or biomechanical (body) position and movement can leave a unique signature confirming that an individual in comparison is one of the same ...*

Mr. Blake stated that he saw approximately 2,000 patients per year in his practice as a podiatrist and that it was highly unusual to see a skeletal structure that has a linear spine without some scoliosis or lordosis, namely one form or another of curvature. He concluded that Otway, in common with the driver of a motor vehicle observed on CCTV, had an unusually erect posture. Mr. Blake estimated that only in about 7% of his practice population did he find 'the slight neck flexion or head poke where the head is projected excessively forward' which he observed in the recordings of the offender and of Otway.

At trial it was argued for the defence that the analysis offered by Mr. Blake could be undertaken by the jurors themselves unassisted by expert evidence ([6] at [11]) and that there was no statistical database against which the jury could judge the

significance of his evidence. In addition, it was contended that there was no scientific basis, and no measurement, to support Mr. Blake's methodology. The trial judge ruled that Mr. Blake should be permitted to give evidence in which he identified the similarities between the walking gait of the appellant and the walking gait of the suspect. It was for the jurors to assess that evidence having viewed the recorded material for themselves. Mr. Blake, however, was not permitted to give evidence relating to the facial features of the appellant or the suspect, and was not permitted to evaluate his comparison of walking gait by reference to the chance that the driver of the motor vehicle at the petrol filling station was someone other than the appellant.

The Court of Appeal received evidence from a professor who was an expert in biology and genetics and who argued that there was an absence of evidence about whether the technique employed by Mr. Blake had been tested in field conditions, subjected to peer review and publication or known rate of error, or subjected to verifiable standards. The Court of Appeal observed that the professor was not a podiatrist and not in a position to express an opinion as to whether Mr. Blake had the expertise which he purported to employ in his analysis. It observed too that in *Re T* [19] at [92]–[96], Thomas LJ giving the judgement of the court, made reference to several cases in which, by reason of the subject matter of the expert evidence, the expert was unable to evaluate their findings by reference to a database of random selection:

> *Nevertheless the evidence of evaluation, founded upon and explained to be the consequence of personal experience, was properly admitted. The proposition that evidence of a comparison cannot be admitted if its evaluation is expressed in terms subjective experience is simply wrong in law (at [20]).*

The Court of Appeal applied the reasoning in *Atkins & Atkins*, a case involving facial mapping evidence in which ([18] at [23]) Lord Justice Hughes stated:

> *An expert who spends years studying this kind of comparison can properly form a judgement as to the significance of what he has found in any particular case. It is a judgement based on his experience. A jury is entitled to be informed of his assessment. The alternative, of simply leaving the jury to make up its own mind about similarities and dissimilarities, with no assistance at all about their significance, would be to give the jury raw material with no means of evaluating it.*

It noted the words of Lord Justice Rose in the 2004 decision of the English Court of Appeal in *R v Luttrell* [21]:

> *"32. For expert evidence to be admissible, two conditions must be satisfied: first, that study or experience will give a witness's opinion an authority which the opinion of one not so qualified will lack; and secondly the witness must be so qualified to express the opinion. The first was elucidated in Bonython [1984] 38 SASR 45, where King CJ (at p.46) said that the question "may be divided into two parts: (a) whether the subject matter of the opinion is such that a person without instruction or experience in the area of knowledge or human experience would be able to form a sound judgement on the matter without the assistance of witness possessing special knowledge or experience in the area, and (b) whether the subject matter of the opinion forms part of a body of knowledge or experience which is sufficiently organised or recognised to be accepted as a reliable body of knowledge of experience, a special acquaintance with which by the witness would render his opinion of assistance to the court.*

> *33. If these two conditions are met and the evidence of the witness is admissible, although the weight to be attached to his opinion must of course be assessed by the tribunal of fact: Robb [1991] 93 Cr App R 161, 165; Darragher [2002] EWCA Crim 1903, [2003] 1 Cr App R 12 para 23 ... It might added that, as with any evidence, expert testimony will not be admitted unless it is relevant in the sense that "it is logically probative or disapprobative of some matter that requires proof": per Lord Simon in Kilbourne [1973] AC 729, 756 D.*
>
> *34. As we have indicated, the appellants argued that evidence should not be admitted unless it passes a certain test, that the evidence can seen to be reliable because the methods used are sufficiently explained to be tested in cross-examination and so to be verifiable or falsifiable. Where, as here, the Crown is seeking to adduce the evidence in a criminal trial, this could properly be considered by the court when deciding whether to refuse to allow the evidence, under Section 78 of the Police and Criminal Evidence Act 1984 or otherwise, in order to ensure a fair trial. We cannot accept that this is a requirement of admissibility. In established fields of science, the court may take the view that expert evidence would fall beyond the recognised limits of the field or that methods are too unconventional to be regarded as subject to the scientific discipline. But a skill or expertise can be recognised and respected, and thus satisfy the conditions for admissible expert evidence, although the discipline is not susceptible to this sort of scientific discipline..."*

Ultimately, the Court of Appeal in *Otway* upheld the trial judge's conclusion that the evidence of Mr. Blake, in the absence of contradiction, was sufficient to establish the existence of (1) the science or expertise; (2) the witness's proficiency in it; and (3) the foundation for the witness's opinion. It accepted ([6] at [22]) the argument that:

> *in a comparison exercise based upon facial mapping or walking gait, it is a necessary condition of admissibility that the witness is able to demonstrate to the court the features of comparison upon which his opinion is formed. Since the comparison is visual, an inability by the witness to explain and demonstrate the features upon which his opinion is formed not only places in doubt the existence of the science or technique claimed; it undermines the foundation of reliability required. We have read a transcript of Mr. Blake's evidence and we have viewed the recorded material from which he demonstrated the foundation for his opinion. We entertain no doubt that the jury was in a position to follow and assess the value of his evidence. There is no danger here that the jury was being invited simply to take Mr. Blake's comparison on trust. We agree with Maddison J, however, that Mr. Blake's ability safely to express his ultimate conclusion in terms of probability of a match, even probability based on Mr. Blake's clinical experience, was insufficiently established. It is important that juries are not misled to an over-valuation of comparison evidence.*

While the Court declined leave to appeal, it was circumspect in its expression of its reasons and concerned not to be seen to be accepting of this novel form of evidence ([6] at [23]):

> *We do not wish it to be thought that we are endorsing the use of podiatric evidence in general. Upon the evidence before him and the argument addressed to this court, we conclude that Maddison J was right to rule the evidence admissible. However, each such application must be considered on its own merits. It may well be, as in the present case, that the trial judge will need to be astute when such evidence is admitted that it is strictly confined within the expertise established and that the*

> *proper limits of evaluation are identified from the outset. We endorse, with respect, the views of the court in T (paras 97–99) as to the necessity for the parties to have issues of disclosure and admissibility well in mind during preparation for the case management hearing so that, when the appropriate time comes, the trial judge is presented with all the material needed to make an assessment of admissibility, and the permissible scope of the evidence if admitted.*

4.2 Hashi v The Queen

In *Hashi v The Queen* [15] a ground of appeal arose from the fact that the prosecution had been permitted to rely upon the evidence of a consultant podiatric surgeon, Mr. Barry Francis, in relation to the similarity of the walking gait of the accused and that of a suspect. He had identified two distinctive aspects of gait – features which he described as an abnormality in the position of body parts in the walking cycle, and flow pattern, which he viewed as a product of the walking cycle, such as its speed or length of stride.

Mr. Francis found six common features between the gait of the suspect and the accused:

1. On the left side the toe was turned inwards;

2. Knee-knock;

3. The stance was upright without head-poke (that is, without the head being thrust forward);

4. There was a long stride with ankle movement in the stride;

5. There was a narrow base of gait (that is, the legs passed close together in the stride);

6. When the suspect turned, he did so from the waist rather than the neck ([15] at [56]).

Of these features the most prominent was the turning inwards of the left foot. Mr. Francis looked for any dissimilarities between the features of the walking gait of the suspect and the accused man in the recordings and found none. He stated that there was no database against which he could make an assessment of frequency and could only refer to his own clinical experience. This led him to express the view that the left-sided inward turn would be found in under 5% of the population. In his view it could only be caused by injury or by one-sided hypermobility, itself an unusual trait. In the absence of trauma, Mr. Francis stated that he would expect to see knee-knock in less than 10% of the population. He stated that there was no predisposition to the presence of one factor in the presence of the other – they were unconnected so it followed that 'a combination of features was more significant than the presence of any one of them ([15] at [57]).

Hashi relied upon the expert evidence of Mr. Blake, the expert who testified in *Otway.* At the relevant time Mr. Blake was the principal podiatrist at the Nuffield Hospital. His view was that the benchmark material was of insufficient quality to attempt an analysis of walking gait.

The Court of Appeal was provided with background material, including published material in relation to the effect of frame rate on the ability to identify

characteristics of gait from CCTV [22] and a Home Office Manual [23] in relation to the screen height on CCTV necessary to discern characteristic detail. It noted that at trial no challenge was made to Mr. Francis' evidence, notwithstanding the view of Mr. Blake. The argument was mounted, though, that Mr. Francis' evidence was unreliable because:

1. he provided his opinion 'in a manner that was overconfident having regard to the material on which it was founded';

2. the material itself was inadequate for the purpose of identifying features of gait;

3. the expert was not qualified to make the 'allowances' he claimed to be able to make and was unable to explain what allowances he had made; and

4. the value of the evidence was overstated by references to population ([15] at [73]).

The Court of Appeal accepted that the quality of the CCTV images 'is at times indifferent' and that care is required to ensure that the feature demonstrated is reliably demonstrated ([15] at [74]). The Court did not accept that Mr. Francis' inability to specify in scientific terms how he made allowances for an imperfect image rendered his opinion unreliable or unsafe:

> *He and the jury were viewing moving and still images. Mr. Francis was showing to the jury the images in which he saw features of gait and either those features could be seen by the jury or they could not. The jury was directed that they could act on the presence of features only if they could observe the features for themselves. Mr. Francis was saying that he had taken account of the imperfection in the image before declaring his identification of a feature. He showed the jury the image on which he relied to make the identification. He was not saying that, although the feature might not be seen by the jury, he, relying on his expertise, was sure it was present. In any instance in which it was argued that the quality of the image was insufficient to be sure that the feature was present the jury had the means to make the assessment for themselves with the assistance of the evidence of Mr. Blake and the cross-examination of Mr. Francis ([15] at [74]).*

This led the Court of Appeal to conclude that with appropriate judicial instructions the jurors could follow the evidence, evaluate its cogency and make their own decision as to whether the features demonstrated were present in each of the recordings. The Court did not agree with the argument that Mr. Francis should not have been permitted to refer to the frequency with which the features or abnormalities he found in the recordings occurred in the course of his clinical practice: 'It was for the jury to evaluate Mr Francis' evidence in the light of the criticisms levelled at him ([15] at [74]). Thus, it concluded that it was not necessary for the judge to have withdrawn the case from the jury at the close of the prosecution case – 'The jury had been provided with the tools with which to make that assessment: in the case of each image on which Mr Francis relied they had their attention drawn to the factors relevant to it, in particular to the quality of the images and the time lapse by which they were recorded. Mr Francis was closely cross-examined on each feature and each significant image' ([15] at [75]).

Both the *Otway* and *Hashi* decisions by the Court of Appeal determined the forensic gait analysis evidence to be admissible and that the risk of inappropriate

juror deference to the evidence was sufficiently addressed by judicial instructions. This was in spite of the evidence in *Hashi* that bore a numerical component of the frequency of the particular attributes in the population seen clinically by the expert. Both decisions were made prior to the intrusion of a reliability prerequisite into English criminal law [2].

5. Australian authority

The most significant Australian authority on forensic gait analysis is the 2020 ruling by Justice Beale in the murder case of *The Queen v Crupi* [16]. Part of the evidence suggesting that Crupi was the murderer was forensic gait analysis evidence given on this occasion not by a podiatrist but by Professor Marcus Pandy, the Chair of Mechanical and Biomedical Engineering at the University of Melbourne. He viewed 95 CCTV clips and was asked to identify and document any physical characteristics viewed in them. He measured certain spatio-temporal features of the gait of the subjects using a Google Earth measurement tool – step length, step frequency and walking speed. He found the step lengths of the subjects were consistently around the same value. In his first report he did not express any opinion as to whether or not the subjects of the clips were the same person but concluded that the mean step length and mean walking speed of the subjects were more consistent with the persons being elderly, as was the degree of toe-out gait – the relevance of this to the prosecution was that Crupi was aged 67 at the time of the murder.

On the basis of the first report by Professor Pandy, he was asked to offer a further opinion as to whether 'a person recorded in any of the CCTV footage contained in clips 1 to 36 is the same as a person recorded in any of the CCTV footage contained in clips 37 to 95'. Police also provided more precise measurements between various geographical features shown in the clips so that Professor Pandy could calculate the relevant spatio-temporal gait features of the subjects more accurately, rather than relying on the Google Earth measuring tool. Professor Pandy concluded that the subjects walked with a gait pattern 'more consistent with that exhibited by older adults, who take shorter steps and walk more slowly than healthy young adults' (at [26]).

The cross-examination of Professor Pandy proved important. He disavowed being an expert on whether persons were similar or dissimilar and said he had never done any research regarding the pitfalls in making such a comparison. He said he was not familiar with the concept of confirmation bias ([16] at [29]). Professor Pandy said he was not a statistician but had sufficient knowledge of statistics to calculate standard deviations. He said there was no way of assessing whether the subjects in any of the clips were walking at their preferred speed and agreed that sometimes he had had to round down the number of steps taken between the relevant locations. He also conceded that he sometimes had to make a judgement as to when a subject's heel struck the ground ([16] at [32]) and that the subjects did not always walk in a straight line between the points. He sometimes observed excessive lateral movement in the gait of the subjects ([16] at [33]).

Professor Pandy said he had never previously been asked to compare CCTV clips of subjects, that he knew nothing about the make or model of the CCTV cameras capturing the relevant images or whether they distorted the images. He conceded he had no information about the frame rates of the CCTV clips and agreed that the frame rates could affect the accuracy of his judgements as to when the heel of the subjects struck the ground (at [34]). Importantly, Professor Pandy was not able to shed any light on the statistical significance of his findings compared with the population at large, and did not purport to do so, instead stating that the mean step lengths of

the subjects in the two groups of clips were 'remarkably similar' ([16] at [35]). He refrained from attributing any specific age estimate to the subjects.

He observed that both feet of the subjects exhibited toe-out gait, although he did not make measurements of it. He stated that it appeared greater than normal for healthy young adults but he did not know the prevalence of a greater than normal toe-out gait in the community ([16] at [37]).

Justice Beale declined to admit the evidence of Professor Pandy. He reviewed the relevance of the evidence by evaluating the potential for a jury to have rationally accepted the reliability of Professor Pandy's calculations. He noted the defects in his evidence about 'rounding-offs' and observed that they may have been concentrated in one group (at [85]) and noted too that Professor Pandy had conceded that the CCTV frame rates, about which he said he knew nothing, may have affected the accuracy of his subjective judgements. He was troubled too that Professor Pandy used the time stamps on the CCTV clip to make his calculations but did not check the accuracy of the various stamps for the various CCTV cameras, as well as the fact that the subjects did not always walk in straight lines between points A and B and that at times there was excessive lateral movement by the subjects. He agreed with the submission from the prosecution that none of these matters may have significantly affected the accuracy of Professor Pandy's calculations but found that 'there is no way of knowing whether that is the case' (at [86]). That led Justice Beale to conclude: 'If the jury is to act rationally, there must be a proper basis for the jury to conclude that the accuracy of his calculations were not significantly affected by these matters. The evidence fails to provide a proper basis for such a conclusion' ([16] at [86]). He was also troubled by what he described as the difficulty of concluding rationally that Professor Pandy was 'comparing apples with apples':

> *He conceded in cross-examination that there was no way of knowing whether the subjects in clips 1–95 were walking at their preferred speed. And yet he based his opinion that the subjects had gait patterns more consistent with older persons primarily on a comparison of the data with results obtained in gait studies of young and old adults whom it was known were walking at their preferred speed ([16] at [87]).*

These issues led him to rule that the evidence of Professor Pandy failed the relevance test under s55 of Australia's 'uniform evidence legislation' and this made it inadmissible. The test in this regard was whether it had the potential to rationally affect the existence of a fact in issue between the prosecution and the defence.

In addition, Justice Beale was at pains to identify the area of specialised knowledge that the prosecution was seeking to adduce. He concluded that it was 'forensic gait analysis of subjects recorded on CCTV footage', this descriptor ultimately being important. He found that the fact that Professor Pandy conceded he was a novice in relation to the task of comparison – whether any of the subjects were the same – and also the CCTV aspect was significant. Professor Pandy conceded that had never undertaken gait analysis using CCTV recordings ([16] at [94]). Justice Beale found Professor Pandy's failure to discuss dissimilarities of gait to be a significant omission in his evidence and that it called into question his expertise to undertake comparative gait analysis. In addition, Justice Beale emphasised that Professor Pandy had conceded that it was the first time that he had conducted forensic gait comparison of subjects recorded on CCTV footage – 'it is difficult to see how Professor Pandy can be considered an expert in that field when this is the first time he has performed the task. For all his undoubted learning and experience,

I am not satisfied that Professor Pandy is an expert in the relevant area of FGA ([16] at [97]). Thus, he found that even if Professor Pandy's evidence passed the test of relevance, it engaged the exclusionary opinion rule ('Evidence of an opinion is not admissible to prove the existence of a fact about the existence of which the opinion was expressed': s76) and was not saved by the exception for opinions based on specialised knowledge (s 79).

Significantly too Justice Beale found that the evidence by Professor Pandy constituted tendency evidence:

> *First, clips 1–36 and 95 show D walking. Second, Professor Pandy's analysis of D's gait in those clips shows that D has a tendency to walk a certain way. Third, because D has such a tendency, he can be expected to walk that way on other occasions. Fourth, clips 37–94 show the shooter walking. Fifth, the way he walks is closely similar to the gait tendency exhibited by D. Taken together, the evidence of D's gait tendency, and its close similarity to the shooter's gait, support P's case that D was the shooter ([16] at [103]).*

This meant that it engaged the exclusionary rules in s97 of the uniform rules of evidence in Australia, requiring evidence to have significant probative value to be admitted. He found that it did not and therefore it constituted inadmissible tendency evidence.

Justice Beale also considered whether the evidence, if it was relevant, which he had found it was not, should be excluded on the basis that its probative value was outweighed by the danger of its constituting evidence that was unfair to the accused. He concluded that the probative value of his evidence was modest because, taken at its highest, it was evidence of similarity, not identity ([16] at [118]). In addition:

> *there is no evidence as to how common or uncommon in the general population are the mean step length, step frequency and walking speed of the subjects in the clips. Further, Professor Pandy does not assert that the subjects of the relevant clips are elderly, just that the spatio-temporal gait features are more consistent with the gait of older persons ([16] at [118]).*

Justice Beale identified two ways in which Professor Pandy's evidence had the potential to be misused – by the jury attaching more weight to it than it deserved, especially in light of his using the phrase, 'remarkably similar' and by reason of the jury engaging in tendency reasoning, a risk that he considered 'very real.' This led him to exclude the evidence as more prejudicial than probative.

6. The Chartered Society of Forensic Sciences and the College of Podiatry Code of Practice

Importantly, in 2020 the Chartered Society of Forensic Science and the College of Podiatry [24] published a 'Code of Practice for Forensic Gait Analysis'. It stipulated that the method used for the preliminary assessment in forensic gait analysis should include assessment of fact such as (but not limited to):

1. distortions of the image inherent in the footage;

2. the resolution (sharpness), lighting and frame rate of the footage;

3. the locomotor activity being undertaken by the figure/subject;

4. the number of consecutive mid gait steps seen in the footage;

5. the position of the camera relative to the figure/subject;

6. the direction in which the figure/subject is moving relative to the camera;

7. the relative size of the image of the figure/subject in the field of view;

8. the possible impact on gait associated with the figure/subject's footwear or lack of footwear; and

9. the possible impact on gait associated with the figure/subject's environment.

The Code also stated that features of gait should only be compared if the locomotor activity being undertaken in the questioned footage is the same as that being undertaken in the reference footage – walking footage should be compared with walking footage. The comparison should consider features of gait that:

a. are exhibited by both the figure in the questioned footage and the subject in the reference footage;

b. would preclude the figure in the questioned footage from being the subject in the reference footage;

c. are exhibited by the figure in the questioned footage, but not the subject in the reference footage, but do not preclude the figure in the questioned footage from being the subject in the reference footage; and

d. are exhibited by the subject in the reference footage, but not the figure in the questioned footage, but do not preclude the subject in the reference footage from being the figure in the questioned footage.

In addition, the Code of Practice ([24] at [18.63]–[18.68]) stated that:

- A database can be used to assist in the determination of the strength of evidence by the forensic gait analysis; however, its admissibility may be questioned if the database does not meet all of the following criteria:
    - ○ It is available for use by both the prosecution and defence.
    - ○ States the size of the population used.
    - ○ States the appropriateness to the case of the population used.
- The likelihood of such a database being deemed admissible may be boosted if:
    - ○ it is also in the public domain; and/or
    - ○ has been peer reviewed and published.

- If a database has been used to assist in the determination of the strength of evidence provided by the forensic gait analysis this shall be made clear in the final report, and the database identified.

- In the absence of the use of a database the final report shall contain a statement to the effect that the determination of the strength of evidence provided by the forensic gait analysis is an opinion based conclusion, and is not predicated on numerical data or statistical calculation.

- Where the expert provides an opinion based solely on their experience it is important that the statement make clear, in detail, the experience which allows the expert to proffer that opinion.

- The strength of evidence provided by the forensic gait analysis should be expressed using a published scale of verbal expressions of strength of evidence in support of one of two opposing propositions. Generally, the prosecution's proposition would take the form of 'the figure in the questioned footage is the subject in the reference footage'. The alternative proposition is determined by the defence but in the majority of cases takes the form of 'the figure in the questioned footage is not the subject in the reference footage'.

The Code of Practice for Forensic Gait Analysis is not binding outside the United Kingdom but it constitutes a sound articulation of responsible practice in a period when the area of forensic gait analysis is moving toward a sounder scientific basis.

7. Status of gait analysis evidence

For the present the area of forensic gait analysis poses significant risks in terms of its reliability and the danger that it will be invested with greater respect than its current scientific status deserves in light of the subjectivity inherent in most of its evaluations and the absence of rigorous databases which might enable a statement of the statistical significance of any finding of similarity.

In addition, it is significant that the quality of CCTV footage varies considerably. This can affect the ability of forensic gait analysts to undertake their evaluations informedly. For instance, frame rates on CCTV footage vary markedly, from one frame every 4 seconds to 25 frames per second. The work of Birch et al. [22] has shown that this variation has a major impact upon the ability of even experienced professionals to identify characteristics of gait with accuracy. While they have commented that, 'Every effort should therefore be made to ensure that CCTV footage likely to be used in criminal proceedings is captured at as high a frame rate as possible' ([22] at 169), this has not resolved the point at which such analysis ceases or commences to be reliable. A further issue potentially affecting such capacity goes to the extent to which the figure targeted occupies screen height [23].

Lynnerup and Larsen [25] have contended that: 'exact photogrammetric measurements may be made from CCTV material of perpetrators and at the scene of the crime, but that care must be taken to ensure that error ranges, especially connected to measuring the human (clothed and masked) body in motion, are critically estimated and evaluated.'

The decisions in the Canadian *Aitken* case and the Court of Appeal decisions in *Otway* and *Hashi* have extended significant and problematic latitude to forensic gait

analysis evidence, regarding the area as one of expertise and sufficient to enable jurors to utilise it as part of their task of evaluating for themselves similarities in different forms of footage of a person of interest and the accused. The ruling by Justice Beale in the Australian case of *Crupi* stands as a contrast to these authorities with his Honour being disinclined to permit opinion of 'remarkable similarity' on multiple bases including relevance, whether the expert was an expert in the relevant area, its constituting tendency evidence, and its being more prejudicial than probative.

A 2017 report by the Royal Society and the Royal Society of Edinburgh, in conjunction with the Judicial College, the Judicial Institute and the Judicial Studies Board for Northern Ireland ([5] at p6) supported the more cautious approach embraced by Justice Beale, warning that:

> *Its underpinning science is sparse and largely translated from the more developed fields of clinical gait analysis and biomechanics, with more recent insights from biometrics. Care is required, however, in assuming that techniques developed in one field can be applied in another with quite different objectives. The scientific evidence supporting forensic gait analysis, as currently practised, is thus extremely limited.*
> *When forensic gait analysis is used as an aid to positive identification of a suspect, the following matters should be borne in mind:*
> *There is no evidence to support the assertion that gait is unique within current or foreseeable limitations of measurements used in forensic gait analysis.*
> *There is no credible database currently that permits assessment of the frequency of either normal or abnormal gait characteristics.*
> *There are no published and verified error rates associated with the current methodology.*
> *There are no published black-box studies of analyst reliability and repeatability.*
> *There is no standardised methodology for analysis, comparison and reporting of gait characteristics.*

Cunliffe and Edmond [10, 26] have been particularly concerned about the current state of the art of forensic gait analysis and of forensic podiatry in general. They have argued powerfully that for the present the evidence is inherently unreliable and urged application of stringent tests to exclude it in most cases from jurors' consideration. They have contended ([24] at 279) that it

> *might be useful as a technology capable of assisting with identification or exclusion. This, however, assumes that technical problems associated with validation, image quality, duration of view, frame rates, different types of movement (e.g., walking, running, dancing, carrying objects, moving with injuries, moving while intoxicated, trying to disguise gait, or walking toward somebody with the intention of shooting), frequency and interrelatedness of features, and cognitive bias can be overcome. As things stand, forensic gait analysis can merely suggest that a person could be included within a set of similar persons where the apparent or alleged similarities are of unknown frequency, so the size of the set is unknown.*

Van Matrigt et al. [11] have correctly identified that: 'To improve clarity on admission of gait as evidence and assessing its evidential value, method validity and reliability and expert proficiency should be reported', a position supported by Cunliffe and Edmond. Van Matrigt et al. [11] have also argued that forensic gait analysts should:

> *join forces to create an international standard forensic gait analysis method with known validity, reliability and proficiency tests. We propose to focus on designing and publishing on large (inter)national gait databases and methods for likelihood calculation taking into account interdependent features. We also hope for (inter) national guidelines for the admission of forensic gait analysis in court. This is especially important since forensic gait analyst is not a protected professional title.*

Macoveciuc et al. [27] have emphasised the increasing role of digitisation of matching procedures within forensic podiatry and usefully argued that: 'A collaboration should be established between the gait analyst and the biometric specialist where the analyst inputs their own expertise to assess the match concluded by the biometric system and also advises (from a clinical and forensic perspective) on the data collection procedures and algorithm development.'

While forensic podiatry in particular and forensic gait analysis have enthusiasts [28] and have the potential to provide assistance to investigators and potentially to the courts, the harder question is as to the appropriateness of forensic gait analysis as expert opinion evidence until further development in the discipline takes place. Undeniably, there are risks in the subjectivity of the comparison process which has the potential to be distorted by cognitive and contextual biases. There is an urgent need for the development of relevant databases so that subjective identification of what is unusual, out-of-the-ordinary or remarkable can be contextualised objectively and, in particular, numerically [29]. Work in this regard has commenced but as yet has some distance to travel. It remains to be seen whether the area remains the principal preserve of forensic podiatrists and whether medical practitioners have a constructive contribution that they can make to its development. In all probability, though, as in a number of areas of forensic science, contributions can be made by practitioners from diverse disciplines, including biometric experts and forensic statisticians, to evaluate the accurate interpretation of results.

For the present what needs to be observed is that there are real questions about the probative value of most forensic gait analysis evidence, that expert evidence in respect of comparison of images needs to be undertaken with temperance and circumspection and that the prejudicial value of such evidence needs to be subjected to rigorous evaluation, including by reference to the Code of Practice for Forensic Gait Analysis published in 2020 by the Chartered Society of Forensic Science and the College of Podiatry [24] to determine whether it should be admitted in criminal trials.

Conflict of interest

The author declares no conflict of interest.

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Chapter 2

Mitochondrial 16S rRNA Gene-Dependent Blood Typing as a Forensic Tool

Hussein O.M. Al-Dahmoshi and Hayder J. Al-Nayili

Abstract

Mitochondrial DNA is an important tool for human identification and is used to differentiate between human and animal blood at the crime scene, because in extreme conditions nuclear DNA is severely destroyed while Mitochondrial DNA contains multiple copies (200–2000) per cell and resists harsh and more stable conditions. Seventy-two blood samples were collected from humans (Homo sapiens), sheep (Ovis aries), goats (Capra hircus), and cows (Bos taurus) (18 blood samples for each). All blood samples were withdrawn by a technician and 5 ml were aspirated using an aseptic technique and transferred to EDTA-Na2 tubes. They were mixed well and stored in a refrigerator. The collection took 2 weeks (May 15, 2019–May 30, 2019). All samples were collected from Al-Diwanyia city. The results of PCR testing revealed that the primer pairs were specific and non-specific products did not appear for all samples. The amplification of Homo sapiens mitochondrial DNA with primer pairs of other (Ovis aries, Capra hircus, and Bos taurus) and amplification of each with primer pairs of another genus gave negative results, and this is primary evidence for primer pair specificity. The amplicon of 16S rRNA gene of Homo sapiens was 1200 bp, Ovis aries was 1060 bp, Capra hircus was 820 bp, and Bos taurus was 1300 bp. The sequencing revealed that no cross-reactivity of designed primer pairs and the PCR assay based on the designed primer pairs will be simple, fast, sensitive, specific, and cost-effective. There is sensitivity, specificity, and accuracy in the designed species-specific primer pairs and applicability of the designed primer pairs in forensics to investigate blood spots or evidence belonging for human, sheep, goat, and cow.

Keywords: *Homo sapiens*, *Ovis aries*, *Capra hircus*, *Bos taurus*, forensic

1. Introduction

Mitochondria possess a small spherical genome, mtDNA, which encodes for the 13 important subunits of the electron transport chain and ATP synthase together with 22 tRNAs and 2 rRNAs necessary for mitochondrial protein synthesis [1, 2]. Mitochondrial DNA presents several characteristics which have the potential to be valuable for forensic studies, especially attendant to the absence of recombination, to a large copy number, and to matrilineal inheritance. Mitochondrial DNA typing founded on sequences of the control region otherwise filled genomic sequence is used to examine a variation of forensic mtDNA

profiling methods used for human proof of identity and present their use in the chief cases of human identification from non-human [3–5]. Mitochondrial markers that are used for species identification are as follows: cytb gene, cytochrome c oxidase subunit I gene, 12S and 16S rRNA segment, and control region in wildlife [6–8]. A short fragment of the 12S rDNA was employed for DNA amplification leading to species identification. The mitochondrial DNA 16S rRNA gene is an advanced genetic marker for animal genetic diversity. Polymorphism sites, nucleotide variation, and haplotype variety were determined using whole sequences of the mitochondrial DNA 16S rDNA gene [9, 10]. Animal mitochondrial DNA (mtDNA) is commonly described as a small, circular molecule that is conserved in size, gene content, and organization [11]. The aim of this study is to design valuable species-specific-PCR tool to discriminate blood of humans from non-human using a species-specific primer design.

2. Methodology

2.1 Study design

The study design was experimental to design species-specific primer pairs for typing the blood samples and their assignment to human (*Homo sapiens*), sheep (*Ovis aries*), goat (*Capra hircus*), and cow (*Bos taurus*).

2.2 Blood sample collection

Seventy-two blood samples were collected from humans, sheep, goats, and cows (18 blood samples for each). All blood samples were withdrawn by technicians and 5 ml were aspirated using an aseptic technique and transferred to EDTA-Na2 tubes and mixed well and stored in a refrigerator. The collection took 2 weeks (May 15, 2019–May 30, 2019). All samples were collected from Al-Diwanya city.

2.3 Primer design

The gene selected for this study is the mitochondrial 16S rRNA gene. The NCBI data base was used to recover the sequences chosen for a primer design. The sequence ID of *Homo sapiens* (NC_012920.1); sequence ID of *Ovis aries* (NC_001941.1); sequence ID of *Capra hircus* (NC_005044.2); sequence ID of *Bos taurus* (NC_006853.1). Primer 3 software [12] was used to design the specific primer using the sequence of above-mentioned sequence IDs. The generated primers were as follows: Homo 16S-F: GCCTGGTGATAGCTGGTTGT, Homo 16S-R: ATCATTTACGGGGGAAGGCG (1200 bp); Ovis 16S-F: AGGCCTAAAAGCAGCCATCA, Ovis 16S-R: GCCCTTTTCTAGGGCAGGTT (1060 bp); Capra 16S-F: GCCTGGTGATAGCTGGTTGT, Capra 16S-R: TCACCCCAACCAAAACTGCT (820 bp); and Bos 16S-F: CTAAGCAGCCCGAAACCAGA, Bos 16S-R: GGGCAGGGTTTTGTGTTGTC (1300 bp).

2.4 Mitochondrial DNA extraction

G-spin™ Total DNA Extraction Kit (50 Preps) (REF: 17045) was used to extract mitochondrial DNA from blood of different species according to the manufacturer's protocol instructions.

Primer	Conditions			References
Homo 16S-F Homo 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		59.3 °C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	
Ovis 16S-F Ovis 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		58.3 °C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	
Capra 16S-F Capra 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		58.3°C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	
Bos 16S-F Bos 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		59.3°C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	

Table 1.
PCR conditions.

2.5 Agarose gel electrophoresis

Agarose gel was prepared by dissolving agarose powder in 1X TBE buffer. The amount of agarose can be dissolved depending upon the purpose in which agarose sheet used. About 0.7% agarose gel was used for visualization of the DNA after extraction while 1.5–2% agarose sheet was used for visualization of PCR product (amplicon). RedSafe (alternative for ethidium bromide) stock solution concentration was 10 mg/ml. Only 5 μl of RedSafe stock solution were added to 100 ml of melted agarose gel to get the final concentration of 0.5 μg/ml [13, 14].

2.6 Primer pairs preparation and PCR conditions

The primers were synthesized at Macrogen/Korea, were provided in a lyophilized form, which were re-dissolved with 300 nuclease-free water according to the institution of the manufacture company to reach to the final concentration (100 pmoles/μl). The working solution will be 10 pmoles/μl to be used directly in PCR [15, 16]. The PCR conditions were calculated using online Protocol Optimize writer software. The conditions were illustrated in **Table 1**.

3. Result and discussion

The four sets of designed primer pairs were submitted to specificity using Primer-Blast and the results revealed that, they are specific to amplify the 16S rRNA gene of humans (*Homo sapiens*), sheep (*Ovis aries*), goats (*Capra hircus*), and cows

(*Bos taurus*) (**Table 2**). The 16S rDNA region is a highly conserved region among mtDNA [17]. mtDNA can be easier to retrieve from low-quantity and/or degraded DNA samples, as it is present at many copies per cell, thus providing a clear advantage over nuclear genome-based methods of species identification [18–20].

The results of PCR testing revealed that the primer pairs were specific and non-specific products did not appear for all samples. The amplification of Homo sapiens mtDNA with primer pairs of other (Ovis aries, Capra hircus, and Bos taurus) and amplification of each with primer pairs of another genus gave negative results. This was primary evidence for primer pair specificity. The amplicon of 16S rRNA gene of Homo sapiens was 1200 bp (**Figure 1A**), Ovis aries was 1060 bp (**Figure 1B**), Capra hircus was 820 bp (**Figure 1C**), and Bos taurus was 1300 bp (**Figure 1D**). PCR amplification and sequence analysis of the mitochondrial 16S rRNA gene was utilized for differentiation/identification and subsequently evaluation of their application in

Gene	Primer sequence 5′ to 3′	Sequence ID of isolate	Identity
Homo sapiens 16S rRNA	F:GCCTGGTGATAGCTGGTTGT R:ATCATTTACGGGGGAAGGCG	MN115376.1 MN053904.1 MN125706.1 MN163828.1 MN163832.1 MN125705.1 MN163282.1 MN125704.1 MN124446.1 MK069579.1	100%
Ovis aries 16S rRNA	F:AGGCCTAAAAGCAGCCATCA R:GCCCTTTTCTAGGGCAGGTT	KP998473.1 KP998472.1 KP998470.1 KP702285.1 MH841968.1 MH841967.1 MH841966.1 MG837554.1 MG837553.1 KU681224.1	100%
Capra hircus 16S rRNA	F:GCCTGGTGATAGCTGGTTGT R:TCACCCCAACCAAAACTGCT	LS992662.1 LS992661.1 LS992659.1 LS992658.1 LS992656.1 LS992655.1 LS992654.1 LS992653.1 LS992652.1 LS992651.1	100%
Bos taurus 16S rRNA	F:CTAAGCAGCCCGAAACCAGA R:GGGCAGGGTTTTGTGTTGTC	EU177866.1 EU177865.1 EU177864.1 EU177863.1 EU177862.1 EU177861.1 EU177860.1 EU177859.1 EU177858.1 EU177856.1	100%

Table 2.
Primer-blast of designed primer pairs.

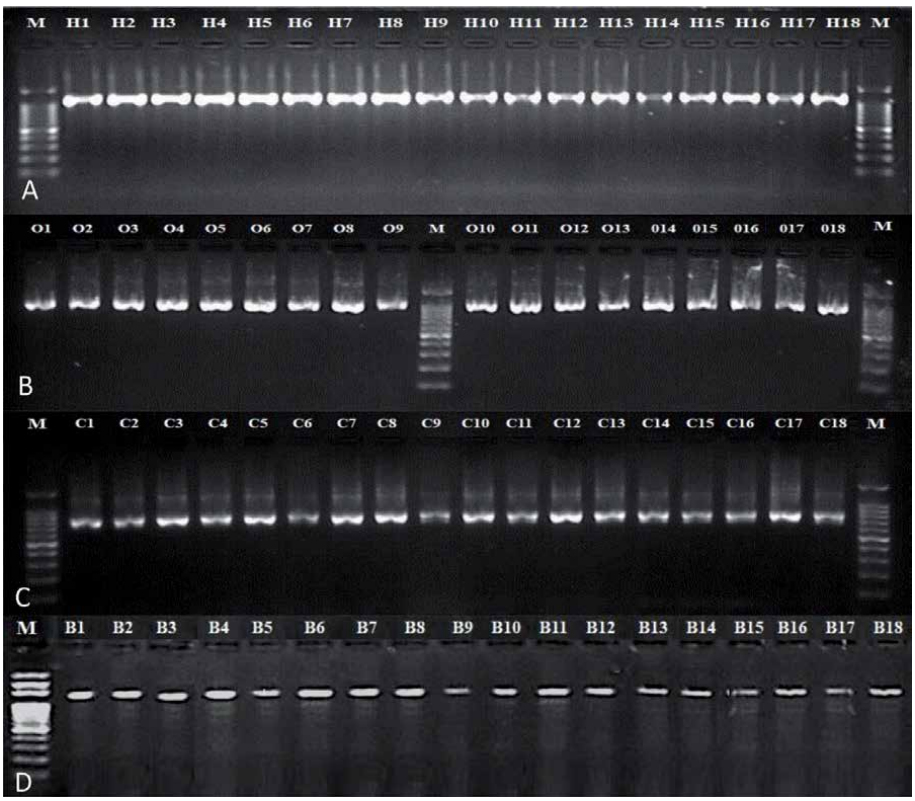

Figure 1.
Agarose gel electrophoresis 1.5% for: (A) 1200 bp amplicon of Homo sapiens 16S rRNA gene. Lanes H1–H18 represent samples. (B) 1060 bp amplicon of Ovis aries 16S rRNA gene. Lanes H1–H18 represent samples. (C) 820 bp amplicon of Capra hircus 16S rRNA gene. Lanes H1–H18 represent samples. (D) 1300 bp amplicon of Bos taurus 16S rRNA gene. Lanes H1–H18 represent samples. M represents 100 bp DNA ladder.

solving forensic cases [21]. Mitochondrial 16S is suitable for the differentiation of 300 mammalian species. The 16S rDNA gene is a common mitochondrial gene for detection of blend mutton and pork at high sensitivity. The mitochondrial 16S rRNA genes have been used as molecular markers to identify mammals, birds, shrimps, and other species using species-specific primers that amplify the 12S rRNA or 16S rRNA gene regions from mtDNA [17, 22]. Gene loci on the mitochondrial genome have been used in species identification. These include the 12S and 16S rRNA loci. The D-loop (displacement loop) has been used less in species identification but more in intraspecies identification. Due to the greater sequence variation at this non-coding locus, it is now being used as a tool for identifying the presence of particular species within mixture of many species [23, 24].

The secondary and confirmatory assay for specificity of primer pairs used in the study was sequences of PCR products. Eight amplicons from each were sent for sequencing using the Sanger technique (Macrogen/Korea). The retrieved sequences firstly must be trimmed to remove unwanted sequences before submitting them for BLASTN. The trimming performed by Bioedit was utilized to obtain the finally processed sequences. Abbreviations of Homo sapiens sequences were used as (HIS-1 to HIS-8), Ovis aries sequences be (IOA-1–IOA-8), Capra hircus sequences (IBCH-1–IBCH-8), and Bos taurus sequences (IBT-1–IBT-8).

The identity percentage and alignment results of the amplified 16S rRNA gene of Homo sapiens, Ovis aries, Capra hircus, and Bos taurus with database were illustrated in **Tables 3–6**, respectively.

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IHS-1	MH444415.1	0.0	98.98%	0/885(0%)	Plus/Plus
IHS-2	MK069579.1	0.0	99.74%	0/771(0%)	Plus/Plus
IHS-3	MK069579.1	0.0	99.57%	2/697(0%)	Plus/Plus
IHS-4	MK069579.1	0.0	99.08%	0/654(0%)	Plus/Plus
IHS-5	MK059695.1	0.0	99.86%	0/701(0%)	Plus/Plus
IHS-6	MK069579.1	0.0	99.39%	0/657(0%)	Plus/Plus
IHS-7	MK295855.1	0.0	99.50%	0/599(0%)	Plus/Plus
IHS-8	MK069579.1	0.0	99.69%	0/653(0%)	Plus/Plus

Table 3.
Identity of blasted isolates (IHS-1–IHS-8) with reference sequences of highest identity percentage.

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IOA-1	MG489885.1	0.0	98.70%	0/769(0%)	Plus/Plus
IOA-2	MG489885.1	4e-170	100.00%	0/329(0%)	Plus/Plus
IOA-3	MG489885.1	0.0	98.98%	0/586(0%)	Plus/Plus
IOA-4	MG489885.1	0.0	99.73%	0/749(0%)	Plus/Plus
IOA-5	MG489885.1	0.0	99.63%	0/542(0%)	Plus/Plus
IOA-6	MG489885.1	2e-157	99.36%	0/312(0%)	Plus/Plus
IOA-7	MG489885.1	0.0	99.17%	2/483(0%)	Plus/Plus
IOA-8	MG489885.1	0.0	99.80%	0/489(0%)	Plus/Plus

Table 4.
Identity of blasted isolates (IOA-1–IOA-8) with reference sequences of highest identity percentage.

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IBCH-1	KP271023.1	0.0	99.02%	0/614(0%)	Plus/Plus
IBCH-2	KP271023.1	0.0	98.58%	0/633(0%)	Plus/Plus
IBCH-3	KP271023.1	2e-174	99.74%	0/378(0%)	Plus/Plus
IBCH-4	KP271023.1	0.0	100.00%	0/729(0%)	Plus/Plus
IBCH-5	KP271023.1	0.0	99.83%	0/595(0%)	Plus/Plus
IBCH-6	KP271023.1	0.0	100.00%	0/480(0%)	Plus/Plus
IBCH-7	KP271023.1	0.0	99.84%	0/618(0%)	Plus/Plus
IBCH-8	KP271023.1	0.0	99.17%	0/481(0%)	Plus/Plus

Table 5.
Identity of blasted isolates (IBCH-1–IBCH-8) with reference sequences of highest identity percentage.

The sequencing of the 16S rRNA has revolutionized the study and identification of human and non-human samples in forensic science. A simple method was developed using universal primers for species identification based on direct PCR sequencing using primer sets that were designed based on the conserved regions of the 16S rRNA loci detected by the comprehensive sequence comparison among 30 animals whole [25]. The mitochondrial DNA method could be a dominant tool for mammalian species identification, especially in forensic cases in which many unidentified biological samples need to be analyzed such as blood spots [25].

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IBT-1	MF169214.1	0.0	99.50%	3/601(0%)	Plus/Plus
IBT-2	KT184455.1	7e-139	100.00%	0/273(0%)	Plus/Plus
IBT-3	KT184466.1	0.0	99.32%	7/1177(0%)	Plus/Plus
IBT-4	KT184466.1	0.0	99.90%	0/979(0%)	Plus/Plus
IBT-5	KT184466.1	0.0	99.90%	0/965(0%)	Plus/Plus
IBT-6	KT184466.1	0.0	100.00%	0/512(0%)	Plus/Plus
IBT-7	KT184466.1	0.0	99.81%	1/1077(0%)	Plus/Plus
IBT-8	KT184466.1	0.0	100.00%	0/1092(0%)	Plus/Plus

Table 6.
Identity of blasted isolates (IBT-1–IBT-8) with reference sequences of highest identity percentage.

The 16S and 12S sequences allow identification of most species to the genus level. Faster-evolving DNA regions are required to identify closely related animal species [26]. The successfully used forensically informative nucleotide sequencing analysis of the 16S rRNA mitochondrial DNA were very valuable to identify before unknown biological specimens of human and animals [27]. The mitochondrial 12S rRNA and 16S rRNA genes, including those from fish and amphibians to mammals including human beings. Therefore, universal primers were designed to amplify sequences in the fast-evolving animal mtDNA [17]. The PCR amplifications of mitochondrial 16S rRNA followed by sequencing and analysis were demonstrated to be very efficient for identification of species origin [21]. The 12S rRNA and 16S rRNA gene sequences of animals reveal the fitting level of interspecific variation but the great level of intraspecific homogeneity [7]. The results showed no cross-reactivity of designed primer pairs and the PCR assay based on the designed primer pairs will be simple, fast, sensitive, specific, and cost-effective.

3.1 Strength of PCR-dependent 16S mtDNA gene

The extraction of mtDNA and amplification of mtDNA genes seem to be accessible, very easy, and cheap. Additionally when the sequences of amplified genes analyzed the results were very clear and no confusion with other genes. The accuracy is very high due to no cross-amplification between species-specific primers that were observed. The sensitivity is also high due to that the mixed blood at a very small amount (10%) can be detected. Their strength over another technique like real-time PCR was, post real time, cannot perform the sequencing when needed for verification.

3.2 Weakness of PCR-dependent 16S mtDNA gene

The only weakness in the technique is requirement for more time, preparation, possibility of contamination, and more machine when compared with real-time PCR.

4. Conclusions

There is sensitivity, specificity, and accuracy of the designed species-specific primer pairs and applicability of the designed primer pairs in forensics to investigate blood spots or evidence belonging for human, sheep, goat, and cow.

5. Registration of sequences in GenBank

All the 32 sequences of the 16S rRNA gene were submitted to GenBank for registration. After checking and revision, the following accession numbers were donated:

16S rRNA homo sapiens (Human): MN192057, MN192058, MN192059, MN192060, MN192061, MN192062, MN192063, MN192064(Appendix 4–69–4–76).

16S rRNA Ovis *aries* (Sheep):MN173528, MN173529, MN173530, MN173531, MN173532, MN173533, MN173534, MN173535(Appendix 4–77–4–84).

16S rRNA Capra hircus (Goat): MN173285, MN173286, MN173287, MN173288, MN173289, MN173290, MN173291, MN173292(Appendix 4–85–4–92).

16S rRNA Bos taurus (Cow): MN197611, MN197612, MN197613, MN197614, MN197615, MN197616, MN197617, MN197618 (Appendix 4–93–4–100).

Conflict of interest

There is no 'conflict of interest' for this work.

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Chapter 3

Forensic Analysis in Wildlife Crime Cases: Microscopy, DNA Profiling and Isotope Analysis

Vivek Sahajpal, Sudhanshu Mishra and Deepika Bhandari

Abstract

Illegal wildlife trade is one of the biggest threats to the environment and biodiversity. The growing volume of illegal trade in wildlife jeopardizes all the conservation efforts across the globe. Many species have become extinct due to the illegal wildlife trade and many have reached the verge of extinction. According to some estimates, the monetary values of the illegal wildlife trade are estimated to be several billion US dollars. To deal with wildlife crime cases, it becomes imperative to have a sound knowledge of the techniques required in the analysis of wildlife crime exhibits. In this chapter, we have outlined the three frequently used techniques in wildlife forensics viz. microscopy, DNA and isotope analysis for addressing the problems of species and individual identification, and additionally identification of the geographical origin of a wildlife sample. The basic essentials of these techniques have been discussed in this chapter.

Keywords: wildlife, crime, forensics, microscopy, isotope analysis, DNA

1. Introduction

Mankind has been exploiting wildlife since times immemorial for basic needs, but the recent commercialization of wildlife trade has decimated some of the species to the verge of extinction [1]. Illegal wildlife trade is one of the biggest threats to the environment and biodiversity. The growing volume of illegal trade in wildlife jeopardizes all the conservation efforts across the globe. Many species have become extinct due to the illegal wildlife trade and many have reached the verge of extinction. According to some estimates, the monetary values of illegal wildlife trade are estimated at around 53 billion USD, and it is globally the third largest illegal trade after illegal trade in narcotics and firearms [2, 3]. Further, some of the wildlife crime and trade have also been linked with other organized forms like funding of terrorist activities, according to the United States Senate Foreign Relations Committee 2009. Studies have revealed that exploitation of wildlife by hunting for trade and pet collection is the second greatest drivers, surpassed only by habitat destruction for the decline in the population of many endangered species, and is impacting mammals (33%), birds (30%) and amphibians (6%) [4]. This has raised a global concern to check illegal trade for conserving wildlife for the future generations of the world and to maintain the delicate ecological balance of the nature.

In this scenario, it becomes the need of the hour to develop wildlife forensics with the changing paradigms of wildlife crime. Some of the important techniques that have made a strong impact in the field of wildlife forensics are microscopy, DNA analysis and elemental analysis, especially the study of isotopes. The aspects of these techniques are discussed in the proceeding sections.

2. Microscopy in wildlife forensics

The spectrum of types of physical evidence in wildlife forensics is very wide and so are the techniques. Microscopy is one of the most useful tools in wildlife forensics, especially while dealing with hair evidence. Hair is one of the important physical evidence found in wildlife crime cases about mammals. Mammals form one of the largest groups of poached species and a large number of wildlife crime cases require identification of species from hair. The history of examination of hair for species identification can be traced back to the nineteenth century, but the first significant contributions in this field were the works of Hausman [5–7] in America. Numerous studies related to species characterization from hair reported in the first half of the last century [5–10].

Microscopic hair characteristics have also been widely used in biological sciences for studying food habits, prey, predator relationships and mammals inhabiting a den or a tree [11–13]. In 1938, Mathiak produced a key to the identification of hairs of mammals of Southern Michigan [11]. In the same year, Williams produced a key to the identification of hairs of moles and shrews [14].

A key for the identification of Californian mammals from hair characteristics was published by Mayer [12]. The entire key was based on a consideration of dorsal guard hairs that had been taken from one small area of the pelage. In all, around 392 species and subspecies were considered. Thoroughly descriptive guides on microscopic hair characteristics of some important mammalian species in certain geographic regions have been worked out by several prominent workers. Guide on hair structure of some selected mammals of Ontario was provided by Adorjan and Kolenosky [15]. Similarly, a guide for species identification from the hair of some selected mammals of Australia was provided by Brunner and Coman [16]. Moore et al. [17] provided a guide for the identification of hair of some mammals of North America (Wyoming). Later guides on species identification from hair were provided by Appleyard [18] and Teerink [19]. Statistical evaluation of quantifiable hair characteristics was also reported. Sato et al. performed a statistical comparison of dog and cat guard hair using numerical morphology [20]. They were able to distinguish between species based on discriminant function analysis. Similarly, Sahajpal et al. used discriminant function analysis to characterize hair from four mongoose species of India, based on the banding pattern of the hair [21]. Sahajpal et al. further reported the guard hair characteristics of four Indian bear species and bovid species listed under Schedule I of Wildlife (Protection) Act 1972 of India [22, 23].

Scanning electron microscopy (SEM) also finds great use in the study of surface morphology of hair and has also been used by several investigators. Rollins and Phan et al. used SEM for the studies of scale patterns in the wool hairs of Ibex, Cashmere/Pashmina and Shahtoosh/Tibetan antelope wool [24, 25]. They were able to show the usefulness of scale patterns of wool fibers for species characterization. A scanning electron microscopy (SEM) study on the cuticular pattern of guard hair of Tibetan antelope (*Pantholops hodgsonii*) was reported by Bahuguna and Mukerjee [26].

It is apparent from the aforesaid facts that examination of hair can provide valuable information on species identification in wildlife forensics. For species characterization of hair, the following aspects are necessary to understand.

2.1 Hair profile

The general shape or profile of the hair has pertinent value in species identification from hair. The hair can be divided into root and shaft. Most of the mammal species have guard hair that flattens toward the distal (away from the skin) end. This flattened region is often referred to as the shield.

2.2 Cuticle

The outer layer of the mammalian hair is made up of scales and is called the cuticle. The layer is very thin and almost transparent. This layer can be considered analogous to the paint on the surface of a pencil. There are three parameters for describing the cuticle:

1. Shape of scale margin

2. Distance between external margins of scales

3. Scale pattern

There are further subclassifications that are beyond the scope of this chapter.

2.3 Cortex

The thick solid layer under the cuticle is called the cortex of the hair. The thickness of the cortex varies across species, and for simple understanding, it can be considered as analogous to the wooden part of the pencil. The cortex is made up of dead cornified cells, packed on to a rigid and homogenous hyaline mass [8]. The pigments that impart a color to the hair are present in the cortex region. Though the cortical region does not have much importance in species identification, the pigment granules present in the cortex do find use in species characterization from hair.

2.4 Medulla

The innermost core of the hair is called the medulla. It can be considered analogous to the graphite lead of the pencil. Medullae have been classified into four basic groups, unbroken, broken, ladder and miscellaneous, based on the general shape, arrangement of cells and air spaces [16, 27]. These four major groups can be further divided into more descriptive categories that cannot be covered in the current chapter.

2.5 Hair cross-section

As discussed in the Section 2.1, hair shows a significant variation in shape across its length. This variation gets very clearly revealed by the outline of their cross-sections. A cross-section of hair shall essentially be circular if the hair has a cylindrical shape. However, for complex shapes, the cross-sections are of very distinct shapes. The cross-section shape and their dimensions are of significance in species identification. For calculating the ratio of medulla and cortex concerning hair thickness, cross-sections are best suited. Cross-sections from the widest portion of the shield are most informative for species identification [19].

2.6 Some important indices

The microscopic hair examination also made use of certain indices that have a significant value in species characterization. The indices find valuable use in statistical analysis. Three indices are commonly used and they are defined as follows:

a. Scale Index: Ratio of the free proximo-distal length of the scale to the diameter of the hair shaft

b. Scale Count Index: Number of scales per unit (1 mm) length of the hair shaft

c. Medullary Index: Ratio of the medullary thickness to the hair thickness (diameter)

2.7 Methods to study hair characteristics

2.7.1 Preliminary examination

Hair samples need to be initially examined for their color, texture, thickness, etc. before microscopic examination. The thickness can be measured in microns using a oculometer on a light microscope.

2.7.2 Examination of cuticle

2.7.2.1 Light microscopy–based method

The almost transparent and very thin layer of the cuticle cannot be appreciated under a transmitted light microscope by using a whole mount of hair. Only the cortex and medulla are visible in the whole mount. Therefore, to view the cuticular structure of hair, a "cast" of hair has to be made and viewed under a microscope. The suitable method is to prepare a cast of the hair. In the case of the hair "scale cast" method, a hair is placed on the surface of a suitable material such that the surface structure of the hair gets reproduced as a three-dimensional cast. This cast can be viewed under a light microscope to observe the cuticular structure of the hair. About 10–20% solution of gelatin in distilled water or 50% solution of polyvinyl acetate in distilled water is used for the preparation of scale casts [28]. A fine and uniform film of the casting media is made on a clean microscopic glass slide with the help of a glass rod or a flat brush in a single stroke along the length of the slide surface. The slide is then placed on a horizontal surface and hair samples are placed one by one on the slide with the help of tweezers. The casting media is allowed to dry for about 20–30 minutes and the hair are removed by plucking gently, leaving behind the three-dimensional cast of the hair surface, which can be used to study the scale patterns, margins and shapes. The cast is viewed under the microscope at a magnification of 100× to 400× depending upon the thickness of the hair. **Figure 1** depicts the scale pattern of the Indian Bison (*Bos gaurus*) under microscope 400× magnification.

2.7.2.2 SEM-based method

Scanning electron microscopy (SEM) is also a recommended method to study the cuticle of hair as it offers resolution much higher than light microscopy. In this method, the hair samples are initially coated with a thin film of gold or palladium under a very low pressure (10–6 Torr) to make the surface conducting. These hair samples coated with a very fine film of gold or palladium are then viewed under

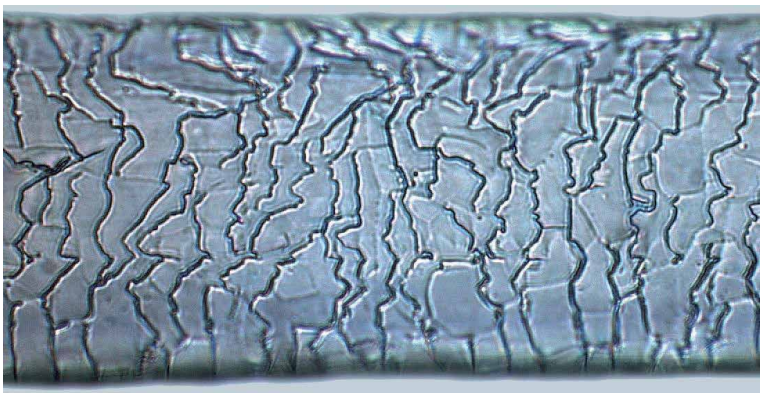

Figure 1.
The cuticle of Indian bison (Bos gaurus) under microscope 400× magnification.

an electron microscope, with the help of an electron beam. The method also has added advantage of studying the elemental profile of the hair if the SEM is coupled with energy dispersive X-ray analysis (SEM-EDXA) or wavelength dispersive X-ray analysis (SEM-WDXA).

2.7.3 Examination of medulla

Medulla can be visualized in the whole mount of hair. However, it is not usually possible to observe the fine structural details of the medulla because of the air filled in vacuoles of the medulla. Hence, it appears as a dark central region when viewed under a microscope. For a proper appreciation of the fine structure of the medulla, the air vacuoles need to be infiltrated with a solvent like xylene. To achieve this, the hair samples are cut into small pieces (0.5 cm to 1.0 cm in length) with a razor blade and immersed in xylene (preferably overnight). These hairpieces after overnight treatment with xylene can be mounted directly on a glass slide in DPX or Canada balsam and viewed under a light microscope. **Figure 2** depicts the Medulla of Serow (*Capricornis sumatraensis*) under microscope 400× magnification. From **Figure 2**, the extent of infiltration by xylene can be clearly appreciated, as medulla

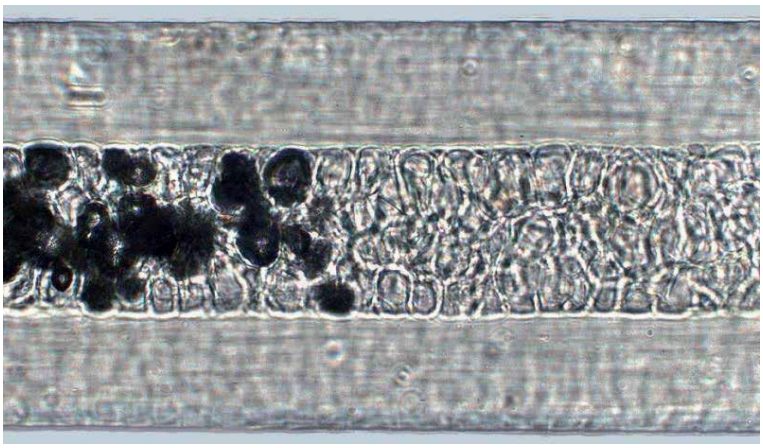

Figure 2.
Medulla of Serow (Capricornis sumatraensis) under microscope 400× magnification (note black globules represent the area where xylene has not infiltrated).

in that part is clearly visible, whereas in the part where xylene has not infiltrated, the medulla shows dark globules. The dark globules can be cleared by increasing the time of keeping the hair cuttings under xylene.

The following four observations can be made:

1. The medulla type

2. Medulla pattern

3. Medulla thickness in microns (using oculometer)

4. Medullary index

2.7.4 Study of cross-sections

The cross-sections of hair can be prepared by using a microtome. In case of nonavailability of microtome, a simple yet reliable method may be used [21]. This method requires a straw pipe, mounting wax and a razor blade for preparing cross-sections. Few hairs were inserted into a straw pipe, keeping them as straight as possible. Maintaining the vertical position of the hair, molten wax is slowly and carefully sucked into the straw. Once the molten wax rises past the hair samples, the straw pipe is constricted to prevent the molten wax from running down. The wax is allowed to solidify and the straw pipe is cut open to remove the wax stubs with embedded hair. These stubs with hair embedded in a vertical plane can be used for cutting fine cross-sections with a razor blade. The cut cross-sections are placed in a microscopic glass slide and a drop of xylene is added to remove the wax. These can be viewed under a light microscope at a magnification of 100× to 1000× depending upon the thickness of the hair. **Figure 3** depicts the cross-section of the hair takin (*Budorcas taxicolor*) under microscope 400× magnification.

The following parameters may be observed:

1. Cross-section outline

2. Medulla outline and configuration

3. Pigment distribution in the cortex

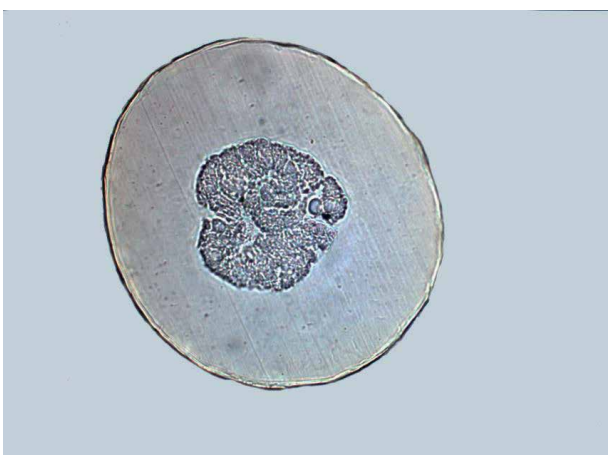

Figure 3.
Cross-section of hair of takin (Budorcas taxicolor) under microscope 400× magnification.

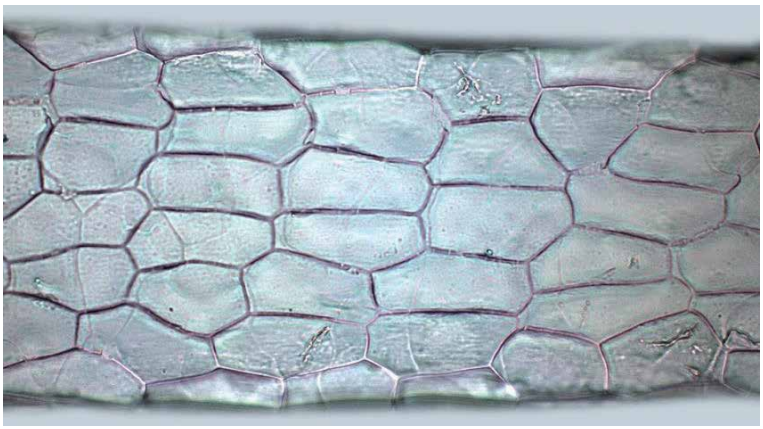

Figure 4.
The cuticle of Tibetan antelope (Pantholops hodgsonii) under microscope 400× magnification. Note the honeycomb-like structure of the scales forensic.

Examination of hair by considering these parameters usually helps to narrow down up to genus and species level. In some species like Tibetan antelope (*Pantholops hodgsonii*), mere examination of the cuticle can help in species characterization due to the unique scale pattern of the cuticle (**Figure 4**). The scales of the cuticle of the hair of Tibetan antelope (*Pantholops hodgsonii*) have a honeycomb-like shape that can be clearly appreciated in **Figure 4**. However, for most of the species, examination of all the parameters is necessary to reach a reliable result.

Sometimes, hair samples may not be in good shape, or may not be in an appropriate number, or may not be available at all. In such cases, it is worthwhile to use DNA-based techniques for species identification. The DNA-based methods are discussed in the next section.

3. DNA analysis in wildlife forensics

In the past 2–3 decades, conservation genetics has evolved as an important tool to resolve problems faced in species conservation. It has wide applications in molecular ecology, population genetics, molecular phylogenetics, taxonomy and phylogeography [29].

A recognized field of conservation genetics, now drawing growing attention, is the advancement of analytical methods to offer strong DNA-based evidence to support conservation law enforcement, which is commonly known as "wildlife DNA forensics." Wildlife forensics is related to the identification of confiscated material to ascertain the species, individual identity or relationship, and source population of the sample. However, wildlife forensics has its challenges. Despite the implementation of national and international laws to protect degrading habitat, protect biological species diversity and secure long-term survival of species, DNA forensics has become a main probing tool to curb wildlife crime [30].

In the past decades, molecular techniques have evolved rapidly allowing forensic researchers to extract genomic DNA from small remains or quantity of biological samples left at the scene of a crime and to establish a connection with the wildlife species and the offender. Forensic scientists have utilized this methodology to monitor the illegal trade of ivory [31–33] and to detect the source population of whale meat confiscated from Japanese markets [34] and Bengal tiger body parts [35]. Wildlife DNA forensics has been proven to be powerful especially in remote

wild areas and the marine environment where poaching of protected or threatened species is tough to detect [36]. This portion of the chapter introduces different methods used in wildlife DNA forensics.

3.1 Methods used in forensic genetic identification

3.1.1 Species identification

The genetic-based analysis is commonly used in wildlife forensics to identify the species from a confiscated item. Species identification is useful in illegal poaching cases to examine the trace amount of evidence from the possession of a suspect or scene of crime [37]. It has also been proven useful in detecting species from shark fins [38, 39], products generally used in wildlife trades such as traditional Chinese medicines (TCMs [40–42], hair [43] decorative items such as ivory idol [44] and burnt samples [45] where morphological identification is not possible or reliable.

Species identification is based on genetic markers that exhibit variation in DNA sequence among species, but are highly conserved or similar within a species [30]. Mitochondrial DNA (mtDNA) is generally preferred as a genetic marker over nuclear DNA (nDNA) for species identification as it is easier to extract from highly degraded and processed tissues or samples. This is because of the presence of multiple copies of mtDNA per cell compared to a single copy of nuclear DNA [46, 47]. Besides, universal mtDNA primers can be utilized to amplify the informative sequence of mtDNA across taxa that are less time-consuming in method development [48]. Polymerase chain reaction (PCR) is especially used for gene amplification [49].

In animals, mitochondrial cytochrome b (Cyt b) and cytochrome oxidase 1 (CO1) genes are commonly used as universal mtDNA markers for species identification [50–58]. The Cyt b gene is a useful mtDNA marker for the identification of several vertebrate species from illegal trade items including seals [59], snakes [60], tigers [32, 56, 61–64], sharks [52], turtles [64] and birds [65].

Sequencing of a fragment (600 bp) of the CO1 gene is highly informative and has been recommended as the inexpensive, fast and efficient approach to characterize species. Researchers around the world are making efforts to utilize the CO1 gene to catalog the entire vertebrate biodiversity on earth (www.barcodinglife.org) [47]. Furthermore, pyrosequencing is another method of DNA sequencing based on "sequencing by synthesis" that facilitates further rapid screening of DNA samples compared to methods used in conventional DNA sequencing [61]. Pyrosequencing can sequence only short fragments (50–500 bp) of DNA, which can restrict its use in DNA forensics unless we target very informative and highly variable regions of a gene [61]. Pyrosequencing has been used to identify twenty-eight European mammal species using very short fragments of 12S rRNA (17–18 bases) and 16S rRNA (15–25 bases) gene regions of mtDNA [66].

However, DNA nucleotide sequencing is a key method followed by comparing sequenced DNA fragments with reference DNA sequences of different species. The similarity or sequence homology between the unknown and reference sequences facilitates to ascertain the species of origin. Moreover, the International Society for Forensic Genetics (ISFG) has approved and validated the use of the DNA sequencing method [67, 68] and validated this method as a method for application in the detection of forensic casework [69]. Furthermore, the important advantage of DNA sequencing is that universal PCR primers can be used to amplify the DNA from unknown or random forensic case samples [56].

Species of unknown samples is assigned by analyzing and calculating the sequence homology with the reference DNA sequences [50, 70] available on DDBJ/EMBL-EBI/NCBI database collaboration (The International Nucleotide Sequence Database

Collaboration, www.insdc.org) and the Barcode of Life Data system (BOLD, boldsystems.org), which is the cloud-based data storage and analysis platform and the part of the CBOL (Consortium for the Barcoding of Life, www.barcodinglife.com).

Another method for identifying species is the construction of a phylogenetic tree. Such tree analysis helps understand the evolutionary relationship between unknown and the reference DNA sequences [56, 71, 72]. Phylogenetic trees allow identifying the reference species as likely source species if it is located closest to the unknown sample. Trees can be constructed using different methods such as neighbor-joining, maximum likelihood, Bayesian and maximum parsimony [73] and in wildlife forensics, there is no consensus over which method to use [74, 75].

Although methods that target single nucleotide polymorphisms (SNPs) other than whole DNA sequences confine their capacity to detect species, it enables researchers to analyze samples that contain multiple species, opposite to DNA sequencing using universal primers [30]. Identification of endangered species from traditional Chinese medicines that may contain plant and animal products has been successfully performed using allele-specific PCR primers and probes [63, 76, 77]. DNA sequencing–based species identification and SNP typing–based ability to examine mixed DNA of multiple species can be mixed [30]. Species-specific primers are used to sequence target species from mixed-species samples like TCMs. This tool has been used to ascertain the body parts of shark [39] and bear bile in TCMs [42].

3.1.2 Identification of the geographic origin

The great concern to wildlife conservation is to ascertain the geographic origin of confiscated items to curtail illegal poaching within the country's boundary and cross-border trafficking of wildlife derivates. In addition to species identification, it is necessary to trace the source population of individual forensic samples to implement wildlife protection laws and CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) regulations. Given this, genetic studies have been widely conducted to infer the source of origin of the poached items and can be used to identify marine stocks harvested illegally. However, few published studies are using these methods in forensic investigations.

Ascertaining geographic origin or source population is based on the ability to assign an unknown or a confiscated sample to its population of origin, counting on the availability of population genetic data from several areas and requiring an adequate genetic differentiation of the source population from other populations. Despite these restrictions, a large number of recent conservation researchers are now emphasizing the urgent need for enforcement methods to efficiently ascertain the geographic origin of samples [30].

In divergent populations, phylogeographic analyses can determine the geographic distribution of the genealogical lineages in which unique haplotypes of mtDNA are correlated with large geographic areas [30, 71]. Generally, the D-loop or hypervariable control region of mtDNA is applied as a genetic marker in ascertaining geographic origin based on haplotypes (individual sequence types of the control region) related to specific populations [30]. This method has been successfully applied to identify the large geographic origins of the Chinese sika deer (*Cervus nippon*) [78] and four species of seahorse (*Hippocampus ingens, H. trimaculatus, H. barbouri* and *H. spinosissimus*) from forensic samples [79].

Furthermore, populations with a subtle genetic variation or with insufficient variation in mtDNA can be identified using population assignment methods by employing nuclear genetic markers that exhibit differences among regions more efficiently than phylogeographic analyses. Population assignment methods are useful in assigning individuals to their source population after testing with all

populations within a large geographic area or landscape complex [35]. Therefore, a strategy to first test individuals with mtDNA haplotypes to identify the wide geographic origin and to second detect a particular source population in its large geographic area where other populations exist can be followed [35].

The frequency of the alleles at hypervariable nuclear DNA (nDNA) genetic marker observed in a natural population can be utilized to characterize population genetic structure and to estimate the probability of an individual or a sample belonging to its putative population of origin. Similarly, a forensic sample is assigned to its probable source population or geographic area [30, 35, 80–83]. The most commonly used hypervariable nDNA genetic markers for population assignment are microsatellites (**Box 1**) and AFLPs (**Box 1**) [47].

1. DNA sequencing

DNA sequencing detects each nucleotide base within a target region of a specific genetic DNA marker. For species identification, DNA sequencing of a fragment (nearly 500 bases) is most commonly utilized to offer species-specific DNA sequence. DNA sequencing facilitates the development of single nucleotide polymorphisms (SNPs), Indels and microsatellites with specific regions of DNA sequence variation.

2. SNP typing

Generally, single bp variations in the DNA sequence at a genetic marker causes differences among species, termed as single nucleotide polymorphisms (SNPs). SNP typing, also known as genotyping, investigates the specific regions with variation in the DNA sequence. SNP typing facilitates cheaper and faster tests that do not need long segments of high- or good-quality DNA but provides less information compared to conventional DNA sequencing. Three most commonly used SNP typing methods in forensics are given below:

2.1 PCR-RFLP

PCR-RFLP (Restriction Fragment Length Polymorphism) utilizes restriction endonuclease enzymes that recognize specific cleavage sites to cut DNA. The resulting nicked fragments are analyzed using agarose gel electrophoresis.

2.2 Allele-specific PCR

PCR primers, employed in the amplification of genetic markers, can be designed for highly conserved DNA regions or fragments (universal primers) or areas where highly variable DNA sequences occur between any species or populations (allele-specific primer).

2.3 Allele-specific probes

In this approach, a combination of universal primers and a specific probe that attaches to a specific variant of DNA sequence is used. Such probes allow detecting the base situated at SNP site.

3. Microsatellite DNA genotyping

Microsatellites or short tandem repeats (STRs) or simple sequence repeats (SSRs) are tandem stretches of 1-6 bp long-short nucleotide sequence motifs (e.g., ATATATAT) that occur randomly and are widely distributed in all eukaryotic genomes [84–86]. Variations in the number of repeat units lead to the difference in the size of both DNA fragments (alleles) that can be resolved and visualized on gel electrophoresis [86, 87]. These polymorphic loci are generally used in genetics and forensics studies. These are codominant markers.

4. AFLP (Amplified Fragment Length Polymorphism)

AFLPs are dominant DNA markers in which an allele is present or absent in an individual. AFLP locus cannot determine the heterozygosity of any individual. Therefore, in contrast to the codominant microsatellite DNA markers, AFLPs have less resolving power to assign an individual to its population of origin [88]; generally, at least 50 AFLP loci and 8 microsatellite loci are recommended to conduct population assignment tests [89]. Furthermore, in comparison to microsatellites, high-quality DNA requirement and greater genotyping errors of AFLPs have proven them to be less versatile [88, 90].

Box 1.
Commonly used techniques in wildlife DNA forensic [30].

Generally, a panel of highly polymorphic microsatellite loci is first selected and used to generate the genetic profile of a test sample or forensic specimen [91, 92]. This profile is assigned to a particular population by matching and comparing its observed alleles with the observed allele frequency in the population. There are several analytical methods available to perform assignment tests [83] and freely available statistical software packages [93]. Population assignment tests are highly

meticulous when the genetic database of all candidate populations has been developed, population or species boundaries are distinct, sampling is random and all population represents Hardy–Weinberg equilibrium (random mating, no inbreeding, the balance between mutation and genetic drift). Conversely, these assumptions are not feasible for several populations, for instance, when population boundaries are not evident or the genetic variations between populations are minimal or low, and populations are small [94]. Where populations are genetically widely distributed, stable isotopes (nongenetic substitutes) may be more appropriate to ascertain the origin of the samples [95].

3.1.3 Individual identification and parentage

For the last three decades, individual identification of forensic specimens based on a unique DNA profile has revolutionized human forensic studies [30]. This technique can be employed to detect the number of individuals used in the commercial market or trade, even from highly processed or powdered products [47]. Baker et al. [96] used a partial fragment of the mtDNA control region (464 bp) and 8 microsatellite loci to identify the minimum number of individual North Pacific minke whales (*Balaenoptera acutorostrata* spp.) from 12 markets.

Highly polymorphic SNP or microsatellite markers are used to generate a DNA profile with a series of gene variants or alleles (**Box 1**). The inclusion of more number of loci or markers reduces the chance that two different individuals will have the same DNA profile. Samples are identified to be from the same or different individuals based on the same or different DNA profiles, respectively. It is important to calculate the probability of identity that two individuals may share the same DNA profile [30].

In Canada, a database of DNA profiles has been established and is commonly used to support forensic investigations of the poaching cases of black bear (*Ursus americanus*), caribou (*Rangifer tarandus*), moose (*Alces alces*), elk (*Cervus canadensis*), white-tailed deer (*Odocoileus virginianus*) and mule deer (*Odocoileus hemionus*) (www.forensicdna.ca/dnadatabases.html). Similarly, DNA profiling has been used to generate strong evidence by matching the blood left on the suspect's knife to the carcass of a wild boar in northern Italy [97].

Furthermore, the ability to validate familial relatedness is also a major application of wildlife DNA forensics. In a forensic investigation, the focus of establishing levels of relatedness lies predominantly on the discrimination of wild-caught animals from captive-bred [30]. Genetic or DNA markers are inherited from both parents from one generation to the next that allows using DNA profiles to validate parent–offspring relationships. Microsatellite loci–based DNA profile database is used in Australia and Europe to authenticate captive bird breeding, whereas parentage DNA analysis is applied to verify caviar of captive sturgeon (*S. Rastorguev pers.* comm.) [30].

4. Study of isotopes in wildlife forensics

Apart from species identification from unknown wildlife sample, quite often it also becomes imperative to identify the geographic origin of the sample. In addition to the DNA-based methods for ascertaining the possible geographical origin of samples, the study of the elemental profiles of samples is also a reliable means for predicting the geographic origin of the samples. This also becomes important when a particular species is protected in one area and not in another, and further when animals from the wild are captured and traded as captive-bred [98]. Among the elemental analysis techniques, a comparison of the ratios of different isotopes using methods such as inductively coupled plasma mass spectrometry (ICP-MS) and

isotope ratio mass spectrometry (IRMS) is an established method for predicting the geographic origin of wildlife samples. Variations in the concentration of elements and ratios of the isotopes have been used widely to ascertain the geographical origins of the African Rhinoceros horn [99–101]. Further, Amin et al. used mass spectrometry to study carbon and nitrogen isotopes and laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS) to measure the relative abundance of isotopes of various elements to ascertain the geographic origin of African Rhinoceros horns [102]. Recently, Alexander et al. used stable isotope analyses to monitor illegally traded African gray parrots [103].

4.1 What are isotopes?

Isotopes are atoms of the same element that have a different number of neutrons in their nucleus; hence, they have the same atomic number but their atomic mass is different. Isotopes can be two types, that is, radioactive isotopes or stable isotopes.

Radioactive isotopes have an unstable nucleus that tends to attain a stable form by emitting radiation. Hence, these isotopes are called radioactive isotopes. The process is also radioactive decay, and during the process, particles and photons are emitted. Carbon-14 (14c), which is widely used in the dating of archeological samples and is a good example of a radioactive isotope. It has a nucleus with six protons and eight neutrons. With time, it decays into the nonradioactive nitrogen-14.

Stable isotopes have stable nuclei and hence do not exhibit radioactivity; that is, they do not undergo radioactive decay. Any element that has isotopes will have a lighter isotope (with a lesser number of neutrons) and a heavier isotope (with a higher number of neutrons). Further, the relative abundance of these isotopes with respect to each other varies significantly with geographical location. Hence, for an unknown sample, if the relative abundance of isotopes of a particular element is determined, it becomes possible to predict the geographical origin of a sample, based on the relative abundance of the isotopes. The elements and isotopes thereof enter the food chain and hence get incorporated into the tissues of living organisms. Further, the elements along with their isotopes remain in the remains of the organisms. As species are distributed according to ecozones and geography, their elemental profile including the relative abundance of stable isotopes is bound to vary with the geographical origins. This is used in wildlife forensics for predicting the geographical origins of wildlife samples with analysis of stable isotopes and elemental profiles. The isotopes most commonly used in forensic science for this purpose are generally H, C, N and O [98, 104].

4.2 Techniques used for isotope studies in wildlife forensics

4.2.1 ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive technique of elemental analysis with a capability to detect metals and nonmetals and very low concentrations. Further, it has the capability of detecting isotopes of an element in a given sample.

4.2.2 IRMS

Isotope-ratio mass spectrometry (IRMS) is a specialization form of mass spectrometry, in which mass spectrometric methods are used to determine the relative abundance of isotopes in a sample.

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4.2.3 LA-ICP-MS

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is a highly sensitive elemental and isotopic analysis method in which analysis can be directly performed on solid samples. It used a process called laser ablation in which a laser beam is focused on the surface to generate fine particles. These particles are then transported to an ICP-MS for digestion and ionization and subsequent detection of elements and isotopes.

Elaboration of these techniques shall be beyond the scope of this chapter. The usefulness of the technique has been demonstrated by several workers in this field and the technology has great potential in identifying the geographic origin of wildlife samples.

As discussed earlier, the spectrum of wildlife forensics is very wide, and to address different queries of investigation, the use of different techniques may be required. Some of these techniques, even though readily available, still may require standardization with respect to wildlife crime samples. The matters get further complicated when the evidence material is very limited; hence, it becomes imperative to have a precise idea of what technique(s) should be employed for the purpose. In this chapter, we have covered some important techniques that may be useful for dealing with wildlife crime cases. However, to get a more refined and working knowledge of the techniques, referring to detailed texts is advised.

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Section 2

Forensic Science

Chapter 4

Reliability and Reproducibility of DNA Profiling from Degraded Samples in Forensic Genetics

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Abstract

Forensic DNA analysis is widely used to determine kinship and the identity of evidence from the crime scene and it is especially important in the identification of human remains after different types of exposure (water, heat, etc.). Currently, there are no official recommendations for forensic scientists as to which bones and tissues are the most reliable among degraded DNA samples. Since 2014 more than 350 fragments of unidentified corpses have been examined in the Forensic Biological Department (Republic Bureau of Forensic Medicine, Kazan, Russia). Based on our experience, the most reliable and reproducible DNA profiles are obtained from lower limber bones (in 90% cases), muscles (in 85% cases) and ribs (in 80% cases). However, we discovered a new source of DNA – the odontoid process of the 2nd cervical vertebra, which contains a high amount of DNA with a better state of preservation than many other bones. According to our results, when a complete skeleton or unidentified corpse is found, it is advisable to provide bones with soft tissue remnants in the absence of deeply embedded putrefactive changes. When working at the crime scene, special attention should be paid to separating small bones and fragments from skeletal remains.

Keywords: Forensic genetics, degraded DNA, DNA profiling, autosomal and Y-chromosome STRs, mitochondrial DNA

1. Introduction

Over the past 20 years, forensic genetic analysis has become the main method for kinship determination and human identification when working with human biological traces and unknown corpses.

DNA identity can be determined 99% of the time (9), but it depends, first of all, on the number and types of studied genetic markers. Traditional forensic DNA analysis is based on autosomal and sex chromosomes STRs polymorphism and sequencing of the non-coding region of mitochondrial DNA. The effectiveness of using these markers varies in different samples due to the state of DNA preservation, especially in samples that have undergone significant changes under the influence of external conditions, such as extreme temperatures (fire), prolonged water exposure or decay in the corpse.

Short Tandem Repeat (STR) analysis is based on DNA typing of specific microsatellite loci with 2 to 7 base pairs length of core units repeat both in autosomal and sex chromosomes.

Due to the high variability in alleles and genotype distribution among unrelated individuals, autosomal STRs DNA analysis serves as an effective human identification tool. The more STR markers are investigated, the more informative individual profiles can be obtained, and the less likelihood coincidence that can be observed. What is more, constant location at specific loci in the genome promotes their stability for a long time even under unfavorable DNA preservation conditions.

Y-chromosomal STRs (Y-STR) are used to determine the presence of male DNA in mixed biological traces. However, the degree of Y-STR preservation is much lower than for autosomal STR and this fact is especially noticeable in the analysis of highly degraded DNA samples. However, Y-STR can be very useful in the determination of paternal lineage relationships and these data can be used as supplementary information to autosomal profiles.

Mitochondrial DNA (mtDNA) markers are significantly better preserved in biological traces compared to autosomal loci (especially in bones) due to their smaller size and multicopy per cell. That is why the study of mtDNA in degraded samples is more often successful. However, there is a significant disadvantage of only mtDNA analysis because of its maternal inheritance. Therefore in order to obtain a complete genetic profile, both types of markers (autosomal and mitochondrial) should be used.

Beside the different stability of described genetic markers, the degree of DNA preservation in biological traces plays a key role in the effectiveness of forensic genotyping and this fact is especially important in DNA profiling of bones and corpse's fragments after putrefactive changes during personal identification analysis [1].

2. DNA profiling efficiency in human remains

Identification of skeletal human remains rightfully belongs to the category of most complicated molecular genetic analysis. This is due, first of all, to the difficulties in DNA extraction from bones with various degradation degree as well as different DNA amount in specific bone cell types [2, 3].

When there is only part of a body, even the smallest bone fragments can be very useful to determine:

1. The fact of the death;

2. The nature of the death; and

3. Relationships with living relatives and so on.

Special traces analysis will assist to answer two questions, but genetic analysis is the only method that allows determination of the kinship between unidentified human remains and probable relatives.

There are various methods and kits for genetic identification that depend on each laboratory's capacity but it is important to obtain all possible genetic marker profiles from the each DNA sample.

For example, obtaining only mitochondrial DNA profile cannot be an objective proof in personal identification because the same mtDNA profile will be found in all maternal relatives, as well as in persons who have a very distant relationship with the deceased. The same applies to the Y-chromosome profile since all male relatives

will have the same Y-chromosome haplotype, as well as men who are very distantly related to the deceased [4].

Therefore, only a complex of molecular genetic profiles will give valuable evidence in skeleton fragments or the whole human body identification, especially when working with degraded DNA. It will allow a significant reduction in the probability error, which in turn, can minimize errors in the probability assessment in identification results [5, 6].

In international expert practice, there are no official recommendations as to what types of bones should be analyzed by preference. It is known that long tubular bones both from lower and upper limbs as well as ribs and teeth more often provided for forensic genetic analysis. But there are several assumptions: first of all, it is not always possible to collect all bone remains from the crime scene and secondly excavated bones may belong to different persons.

Over 350 fragments from unidentified corpses were analyzed in our laboratory. Our data show that ribs and their fragments were more often sent for genetic analysis (114 cases (32%)); in 83 cases (23%) lower limbs (mainly femurs) were analyzed. The bones of the upper limbs (29 cases/8%), teeth (21 cases/6%) and skull fragments (15 cases/4%) were also found suitable for genetic analysis. Only a few cases of sternum, clavicle, vertebrae, phalanges, heel bone, a spongy bone fragment, pelvic bone and liver (total 8% of all cases) were analyzed.

Soft tissues can also be an additional source of DNA. According to our data, muscles usually go along with bones and in very rare cases as independent objects because of its quick destruction due to putrefactive changes (**Figure 1**).

Muscles were examined in 69 cases (19%), and what is more, in 49 cases, tissues were chosen by an expert-geneticist. However, it is noteworthy that in all these cases a stable genetic profile was obtained.

The reliability of DNA profiles in human remains varies significantly. According to Jakubowska et al., DNA is better preserved in the sternum (90–100% recovery), in lower extremities bones (80–90%), in the lower jaw and teeth, worst - in pelvis, ribs and shoulder girdle bones [7]. These data are somewhat contradictory because different laboratories use their own equipment and reagents for DNA extractions that can affect the final DNA profiling results. Besides, the degree of DNA preservation in bones is influenced by many physical and chemical factors both of exogenous and endogenous nature [8]. In addition, bone cells - osteocytes – carrying basic genetic information, are located unevenly along the length of the tubular bone. That is why the amount of DNA and its state of preservation would not correlate with the bone type [9].

Herein, we describe the results of our experience for genetic profiling of different bone types and tissues.

2.1 Ribs

Stable genetic profiles were obtained in more than 80% of analyses of ribs and their fragments. Moreover reliable results were obtained in DNA samples extracted

Figure 1.
Femur with the fat wax indicating muscle tissue degradation.

from rib fragments after fire and in exhumed corpse bones. In 2% of cases a partial profile was obtained, which made it possible to compare the unidentified corpses with close relatives. In 18% of studied cases DNA profile was not determined.

2.2 Lower limb bones

As noted above, femurs were the most frequent type of bones for DNA analysis; rare cases were presented by the tibia. In three cases, bone fragments (cuts) from lower limbs were provided without specifying their localization. Stable genetic profiles were obtained in 90% of cases of examined bones.

It i's significant tooworth noting that in 30% of cases of lower limb bones genotyping, STR profiles were obtained only after repeated DNA extraction using the same or different extraction methods.

2.3 Upper limb bones

In most cases, bone fragments were presented by the humerus as well as clavicles, radial bones and ulna. Positive results were obtained in 65% of cases.

2.4 Teeth

A stable genetic profile was obtained in 61% of cases and 39% of cases were not successful. Moreover, in seven of the positive cases, the profile was obtained cumulatively while studying other bones from the same skeleton. Several types of research show that teeth are the preferred source for DNA extraction [10, 11]. However, the methods proposed differ from those used in our laboratory which may affect the low percentage of obtained stable profiles.

2.5 Skull fragments

Skull bone fragments and whole skulls were provided in only 15 cases. Moreover, the whole skull was presented for analysis in only five cases. A stable genetic profile was obtained in 54% of cases, mainly when analyzing the mastoid process and mandible.

2.6 Other fragments

Out of 7 cases of sternum analysis, only three cases gave positive results in determination of stable autosomal STR profile. There have been several cases of examination of fragments of the lung, cancellous bone, calcaneus, vertebrae (without specifying localization). The profile was obtained in all indicated cases. When examining the bones of the pelvis and phalanges of the fingers, the genetic profile has not been established.

Thus, a stable genetic profile was often obtained in long bones of upper and lower limbs and ribs compared to the other bone fragments (**Table 1**).

2.7 Muscles and tissues

A stable genetic profile was obtained in more than 85% of cases in tissue analysis. However, negative results in most of the unsuccessful cases were associated with the circumstances of the accident (heating or chemical exposures) which led to DNA damage. In such cases, bones with/without soft tissues remnants were provided for analysis to obtain possible DNA profiles.

Storage conditions, Death duration	2nd cervical vertebrae (odontoid process)	Femur	Ribs	Teeth	Elbow	Brachial bone	Skull bones
Forest, 2 years	100	100	0	90	—	—	—
Forest, 4 years	—	80	—	—	30	—	30
Forest, around 10 years	80	80	0	50	—	—	40
Exhumation after 10 years burial	—	90	20	—	—	50	—
World War II victims (over 78 years), 1–2 m under the ground	80	—	30	50	—	—	—
Water, 2 years	100	80	—	80	—	60	—
Water, 12 years	100	—	—	80	—	—	—
Field, 1 year	100	100	100	—	—	—	—
Heating track ($+80^0$C), 2 years	0	0	0	—	—	—	—

Table 1.
Effectiveness of autosomal STR loci analysis (%) in different bones (based on AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, USA)).

In two cases it was not possible to obtain a genetic profile of almost fresh muscles without any signs of putrefactive changes even after using different DNA extraction methods (classical phenol-chloroform extraction; magnetic particles extraction (PrepFiler Forensic DNA Extraction Kit® (Applied Biosystems, USA) and ion-exchange columns (QIAamp DNA Investigator Kit, Qiagen, USA)). These cases were not related to each other and they did not coincide in time. However, common factors that may influence the effectiveness of genetic analysis were hectic lifestyle and abuse of low-quality alcoholic beverages and alcohol substitutes (as it was determined later by bone identification).

It is also difficult to obtain genetic profiles from muscles after a fire but our practice shows that good results can be obtained when examining different tissue types. For example, we determined a stable DNA profile in 5 cases of genetic analysis of liver fragments. Therefore, forensic scientists should be very careful when choosing muscles for DNA analysis and, if it is possible, to duplicate results DNA profiles from bones and other tissues.

A stable genetic profile can be obtained in unexpected for DNA analysis samples. And in contrary, bones or muscles that seem to be quite appropriate for DNA extractions at first glance may turn out to be unsuitable for getting DNA profiles as further described in several practical cases.

For example, a fragment of a tubular bone with soft tissues remnants (**Figure 2**) and a second cervical vertebra (**Figure 3**) from the corpse of an unidentified woman were provided for analysis. The duration of death was no more than 5 days and no putrefactive changes were noted.

At first glance, the objects seemed to be quite suitable for research. After DNA extraction with PrepFiler Forensic DNA Extraction Kit®, DNA concentration was measured by qPCR with Quantifiler Human DNA Quantification Kit® (Applied Biosystems, USA).

Figure 2.
Tubular bone fragment with soft tissue remnants.

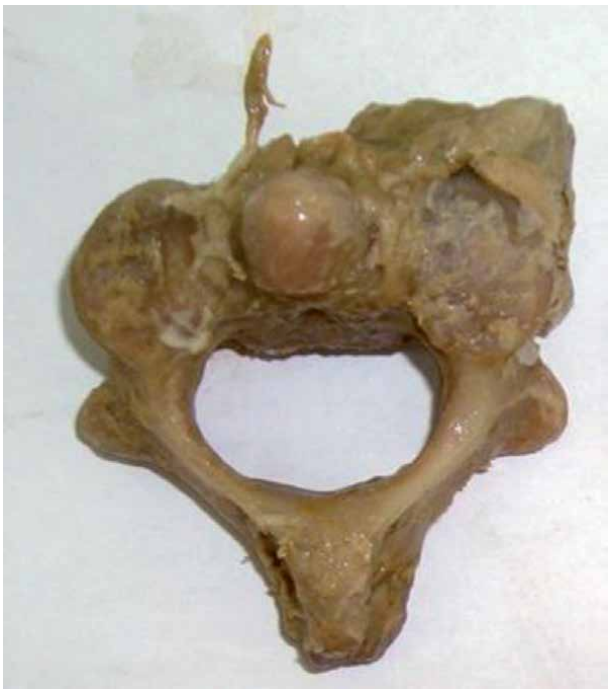

Figure 3.
2nd cervical vertebrae.

According to the PCR results, no DNA was detected in muscle fragments, but DNA concentration in samples extracted from both tubular bone and the odontoid process of the 2nd cervical vertebrae was sufficient for further autosomal STR analysis (0.054 ng/µl and 1.5 ng/µl respectively). However, in spite of sufficient DNA concentration, additional amplification peaks for more than 2 alleles were obtained in some short length loci due to probable stutter effects (**Figure 4**) and normal one and two alleles genotypes were detected for other markers.

Stutter effect can be due to the DNA degradation and the predominance of amplification of shorter fragments that was proved by another qPCR method. We determined DNA quantification and degradation degree estimation by the Quantum DNA-Set kit (Eurogen, Russia) by measuring the concentration of different DNA fragment lengths (91 bp, 156 bp and 211 bp) and their ratio. It turned out that the studied DNA was predominantly presented by the short fragments up to 156 bp length, and their content exceeded the content of long fragments (up to 211 bp) at least three times. After that a number of new DNA extraction experiments were carried out and finally a robust genetic profile was obtained.

In contrast to what was said above, a stable result can be obtained in objects that at first glance are unsuitable for research. Thus, the remains of an unidentified

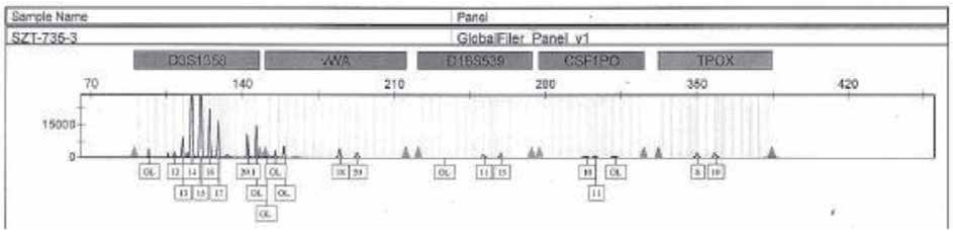

Figure 4.
Partial electrophoregram of multiple alleles genotypes for autosomal STR-typing the DNA extracted from 2nd cervical vertebrae.

Figure 5.
Fragments of blood vessels after a fire.

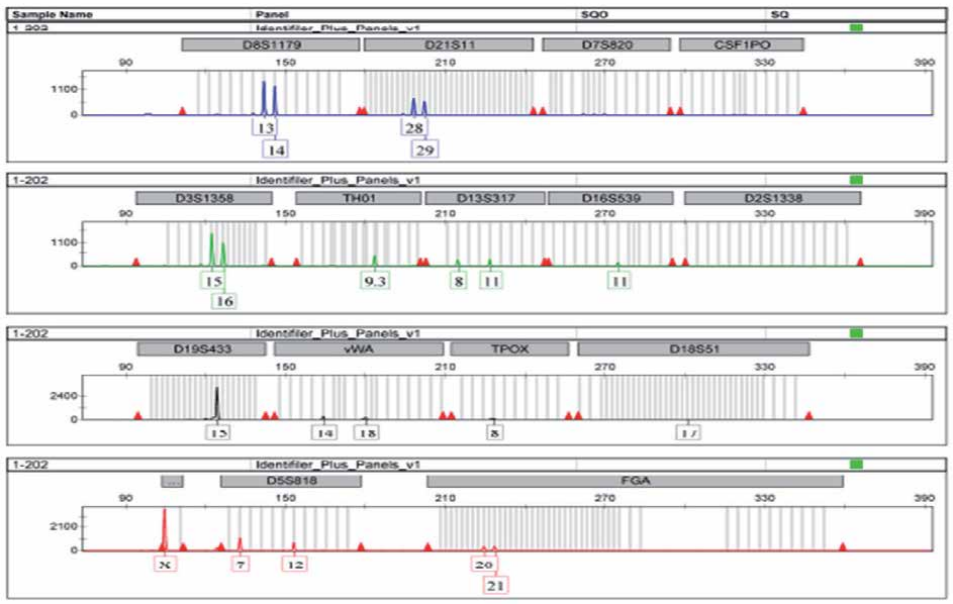

Figure 6.
The DNA profile of autosomal STR loci in DNA sample extracted from burnt blood vessel.

body, discovered after a fire, were delivered to the laboratory. Most of the body was burned out completely (bones were burned out to the point of white heat; the skull was destroyed). In addition, body fragments were exposed by water and

special agents during extinguishment of the fire. However, in the abdominal and chest cavities, a fragment of a large blood vessel with elements of clotted blood was discovered (**Figure 5**).

Despite the obvious degradation, DNA was isolated by the method of magnetic particles and appropriate concentration was determined (0.18 ng/μl). What is more, the female gender was verified and a partial genetic profile was obtained (**Figure 6**). Further identity determination was continued by the comparison with the mtDNA sequence of the person suspected to be the biological son.

Considering the fact that only a partial profile was obtained for autosomal STRs, hypervariable regions of mtDNA were analyzed both in the blood vessel sample and person suspected to be the son, which showed complete a match in both samples so the identification of unknown corpse was determined by two types of DNA markers.

3. Examples of DNA-profiling in certain cases

3.1 Case #1

The skeletonized human remains from the burial of an unknown soldier from World War II (1941 was presumed to be the year of death) were delivered to the forensic biological department of the Republic Bureau of Forensic Medical Expertise. Skeletal remains were represented by a fragment of the humerus, clavicle, a fragment of the temporal bone, the second cervical vertebra and three molars (**Figure 7**).

Bone and teeth powder (300–400 mg and 100–150 respectively) was used for DNA extraction.

DNA was extracted with PrepFiler Forensic DNA Extraction Kit® (Applied Biosystems, USA) using the standard bone extraction protocol according to the

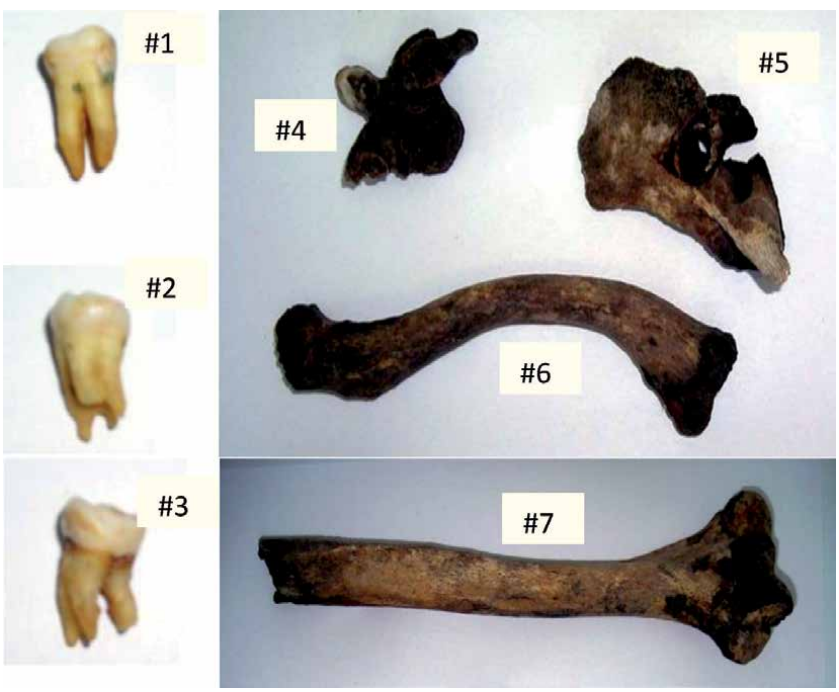

Figure 7.
Bone fragments and teeth from the victim burial were provided for DNA analysis. 1–3 –teeth, 4 – 2nd cervical vertebrae, 5 – Humorous bone fragment, 6 – Clavicle, 7 - temporal bone fragment.

manufacturer's instruction with several modifications. The bone and teeth powder was preincubated for 18 hours at 56°C in PrepFiler BTATM Lysis Buffer (Applied Biosystems, USA) with the addition of DTT and proteinase K before the main extraction procedures.

DNA concentration, measured by qPCR with Quantifiler Human DNA Quantification Kit® (Applied Biosystems, USA), was sufficient for further analysis in two teeth (objects 1, 2) and odontoid process of the 2nd cervical vertebrae (object #4) (0.03 ng/μl and 0.021 ng/μl respectively). DNA concentration extracted from 3rd tooth (object #3), humorous fragment (object #5), clavicle (object #6) and temporal bone fragment (object #7) turned out to be below the lower limit of 0.01 ng/μl. For this reason the samples were set aside for further DNA analysis.

DNA profiling was performed by both autosomal and Y-STR loci with AmpFLSTR® Identifiler® Plus PCR Amplification Kit and AmpFLSTR™ Yfiler™ PCR Amplification Kit (Applied Biosystems, USA) according to the manufacturer's instructions.

Individual DNA profiles on autosomal STRs were detected both from tooth #1 and tooth #2 (**Figure 8**) and were found to be identical to the genotypes determined in STR typing from the odontoid process. (**Figure 9**).

In addition, similar Y-chromosome DNA profiles were obtained from these DNA samples (**Figures 10** and **11**).

It is known that DNA is not well preserved in the vertebrae. However, the odontoid process of 2nd cerebral vertebrae is a newly discovered DNA source with a better state of preservation and higher effectiveness for further DNA analysis as already described in our previous work [12].

3.2 Case #2

A hair sample was provided to our laboratory for comparison with a possible suspect's DNA profile. According to morphological examination, hair belongs to the group of regional human hairs from the upper extremities. The hair follicle was partially turned off but the vaginal membranes were preserved.

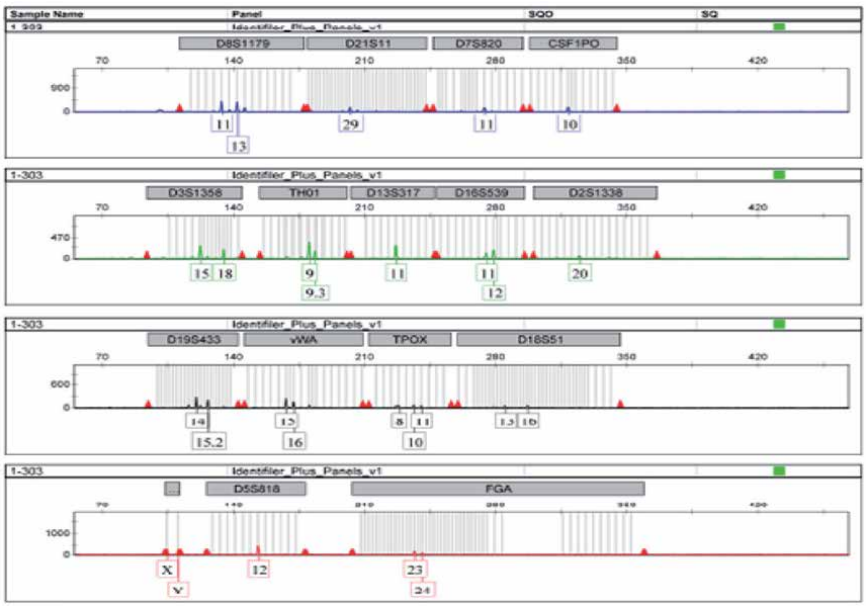

Figure 8.
Autosomal STR profile from object #1 (tooth).

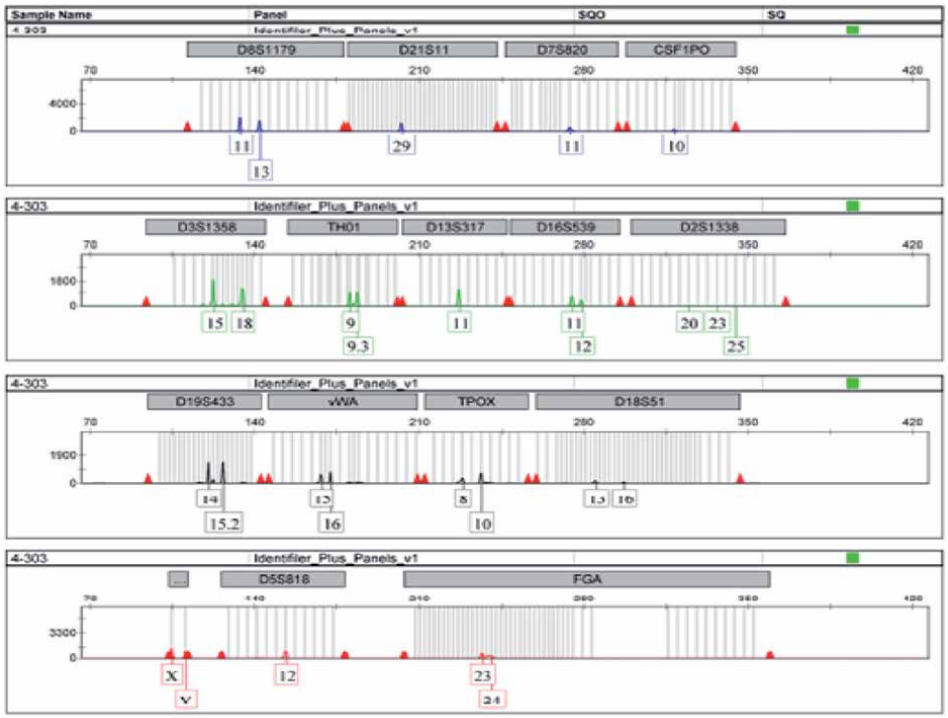

Figure 9.
Autosomal STR profile from object #4 (the odontoid process of 2nd cervical vertebrae).

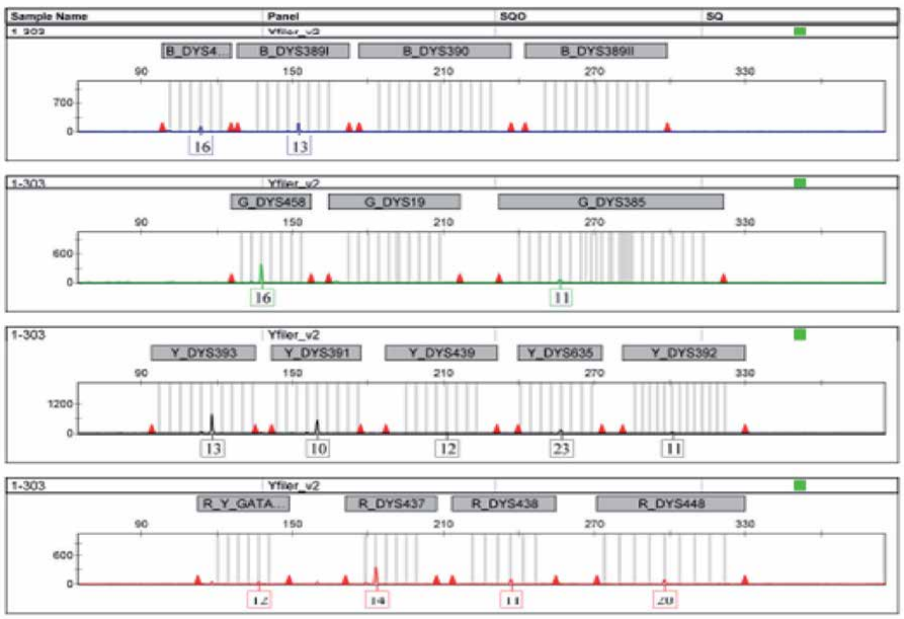

Figure 10.
Y-STR profile in DNA sample extracted from the tooth (object #1).

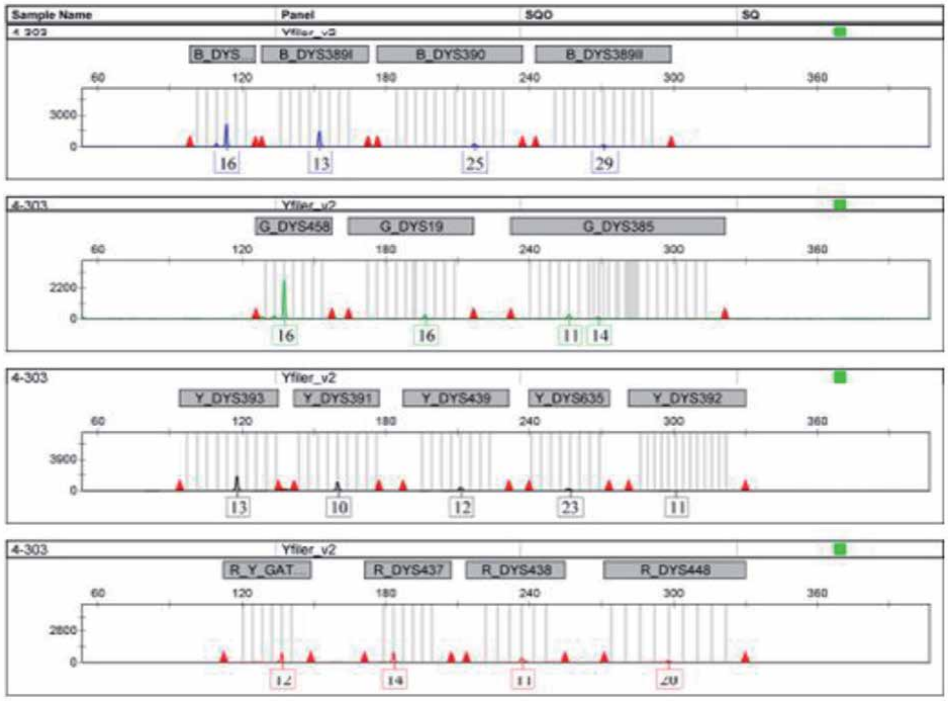

Figure 11.
Y-STR profile in DNA sample extracted from the odontoid process of 2nd cervical vertebrae (object #4).

DNA extraction was performed from bulb using Chelex-100 in order to avoid DNA loss and DNA concentration was measured by qPCR using the Quantifiler Human DNA Quantification Kit® (Applied Biosystems, USA) which was showed to be outside the lower detectable limit (0.009 ng/μl) for autosomal STR amplification. However, we attempted to establish an autosomal genetic profile. A profile was partially obtained and the male gender identity was verified (**Figure 12**).

The identified loci were found to be matched with one of the suspects; however, the data obtained were not sufficient for the identification process. Accordingly, we analyzed hypervariable regions I and II of the mtDNA D-loop (HVS-1 and HVS-2) with MitoPlex system (Gordiz, Russia).

Individual sequences from hair were obtained (**Figure 13**) which that was it made it possible fr there to be a comparison to compare with the suspect's mtDNA sequence (**Figure 14**).

According to these results even one hair with a partially preserved bulb can be sufficient to obtain an incomplete autosomal STR profile which can be supplemented with a profile of mitochondrial DNA to enable a comparison with the DNA of suspects.

3.3 Case #3

The laboratory received biological materials from a crime scene after a quarrel between two men who had been drinking alcohol. One of them committed the murder of the other. The offender tried to hide the traces of the crime by the dismemberment of the body and attempted to destroy the traces by burning them. However, he did not take into account that the human body cannot be burned

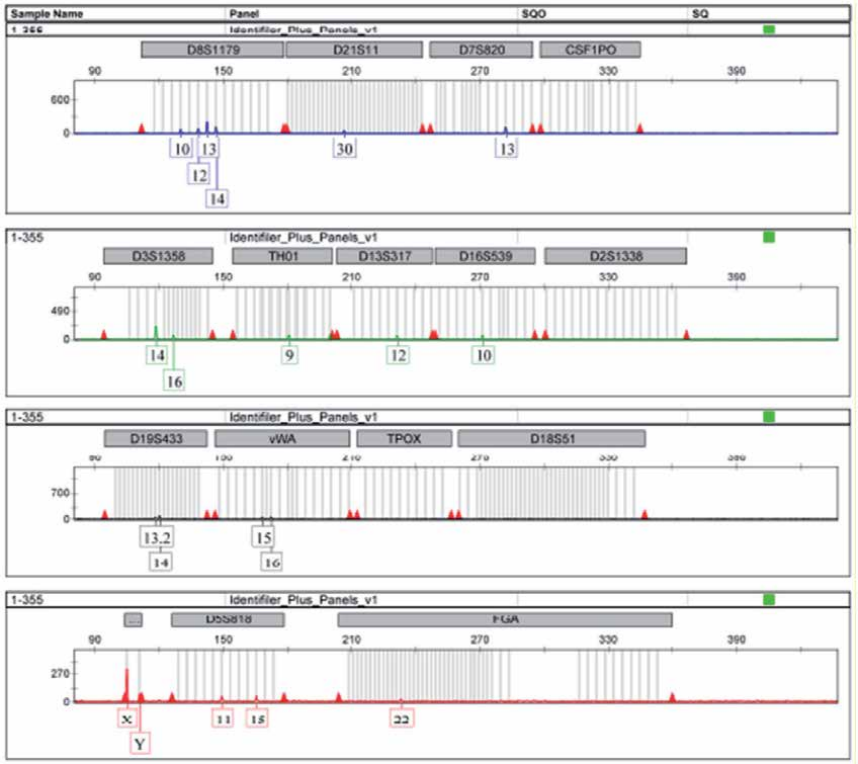

Figure 12.
Partial autosomal STR profile obtained from the hair bulb.

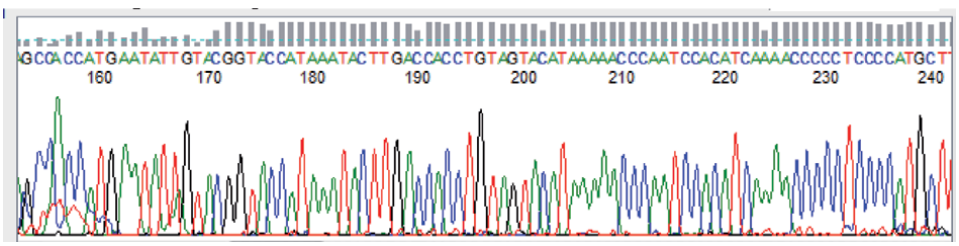

Figure 13.
Part of HVS1 sequence for DNA sample extracted from hair bulb.

quickly. He tried to pull the body out of the fire by the lower limbs, but the upper limbs and head remained in the pit and continued to burn. Then the suspect threw the lower limbs with a part of the pelvic girdle into the river where they were found a day after. The victim and the criminal were identified.

To identify the deceased person, it was necessary to clarify several circumstances based on the morphological and molecular genetic analysis:

1. whether objects discovered at the fireplace were tho0se of a human;

2. to identify the anatomical origins of the remains;

3. to establish the identity of the remains discovered in the river;

4. to prove the identity of the missing person's genetic profile using the profile obtained from the items of clothing

```
CLUSTAL format alignment by MAFFT (v7.475)

hair_HVS1       acggtaccataaatacttgaccacctgtagtacataaaaacccaatccacatcaaaaccc
suspect_HVS1    acggtaccataaatacttgaccacctgtagtacataaaaacccaatccacatcaaaaccc
                ************************************************************

hair_HVS1       cctccccatgcttacaagcaagtacagcaatcaaccttcaactatcacacatcaactgca
suspect_HVS1    cctccccatgcttacaagcaagtacagcaatcaaccttcaactatcacacatcaactgca
                ************************************************************

hair_HVS1       actccaaagccacccctcacccactaggataccaacaaacctacccacccttaacagtac
suspect_HVS1    actccaaagccacccctcacccactaggataccaacaaacctacccacccttaacagtac
                ************************************************************

hair_HVS1       atagtacataaagccatttaccgtacatagcacattacagtcaaatcccttctcgtcccc
suspect_HVS1    atagtacataaagccatttaccgtacatagcacattacagtcaaatcccttctcgtcccc
                ************************************************************

hair_HVS1       atggatgacccccctcagataggggtcccttgaccaccatcctccgtgaaatcaatatcc
suspect_HVS1    atggatgacccccctcagataggggtcccttgaccaccatcctccgtgaaatcaatatcc
                ************************************************************

hair_HVS1       cgcacaagagtgctactctcctc
suspect_HVS1    cgcacaagagtgctactctcctc
                ************************
```

Figure 14.
Multiple alignment of mtDNA HVS1 sequences in DNA samples extracted from hair and suspect's blood using MAFFT program (https://mafft.cbrc.jp/).

However, there were difficulties in identification of the victim because of the absence of a whole body and in addition, the alleged deceased did not have any relatives other than a sister. By way of biological material relating to the deceased man, items of his clothing with his traces were removed from his house (panties, T-shirt, hat, gloves).

Forensic scientists recovered several charred objects of biological (human) origin from the fireplace such as liver fragments (object 1) and finger phalanges (object 2) for further DNA analysis (**Figure 15**).

DNA was isolated from these objects, as well as from the lower limbs recovered from the river and clothes of the missing man using the PrepFiler Forensic DNA Extraction Kit® (Applied Biosystems, USA).

The DNA concentration was measured by qPCR using Quantifiler DUO DNA Quantification Kit® (Applied Biosystems, USA). By using Quantifiler DUO it was possible to prove that the lower limbs, burned liver fragments and finger's phalange belonged to a man.

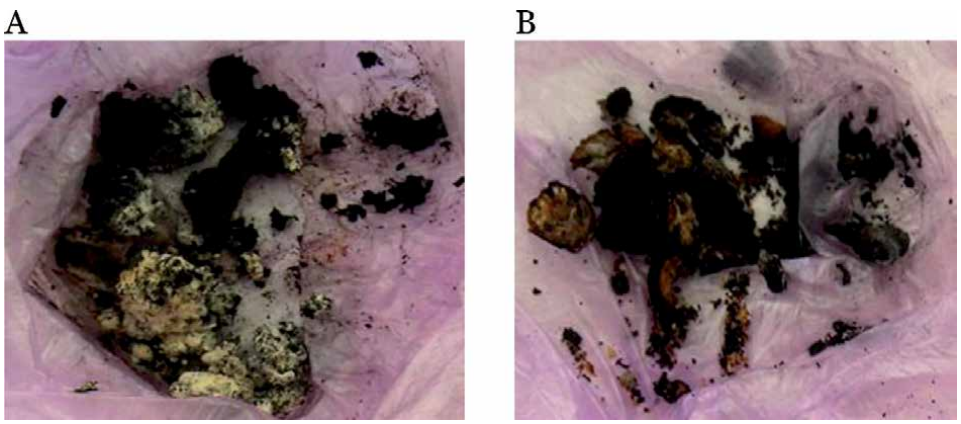

Figure 15.
A. Burnt liver fragments; B. burnt finger phalanges.

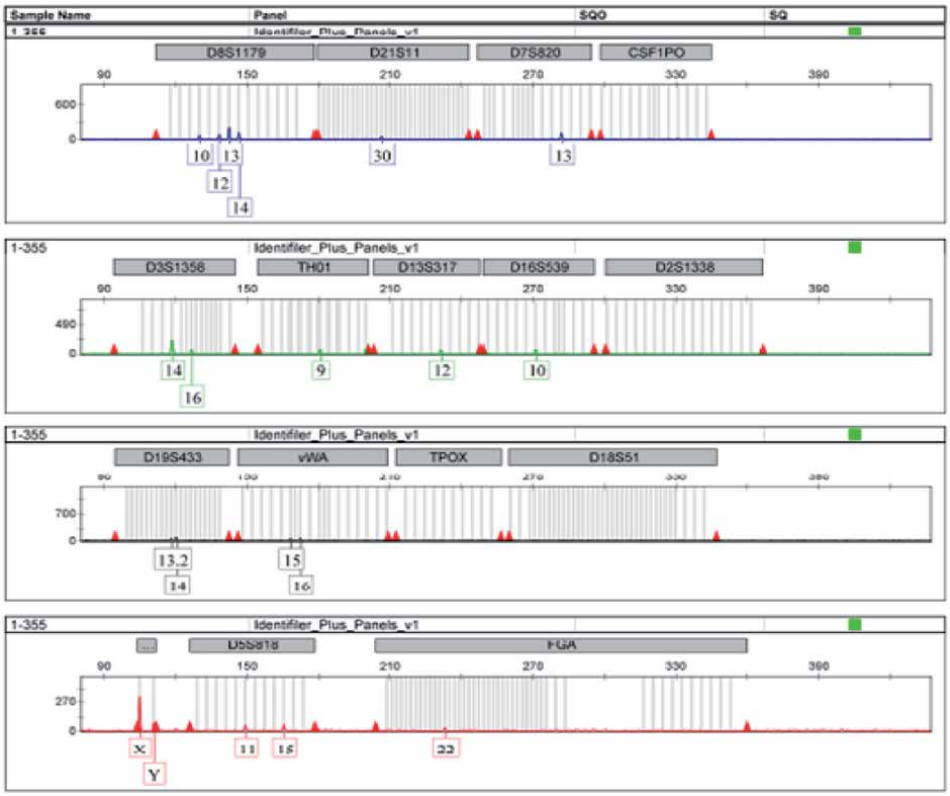

Figure 16.
Autosomal STR profiling from the burnt liver fragment.

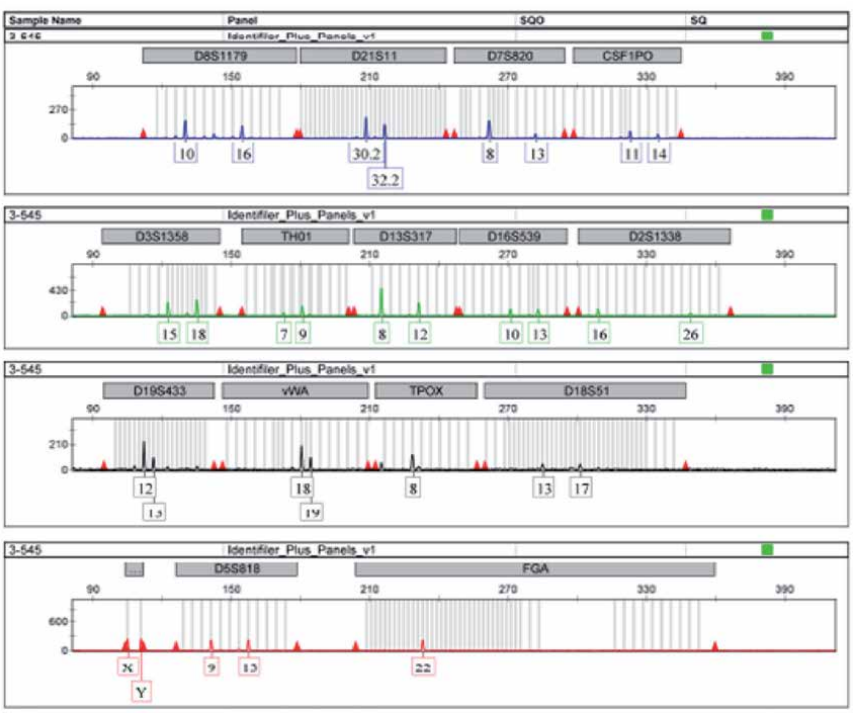

Figure 17.
Autosomal STR profiling from the burnt phalange.

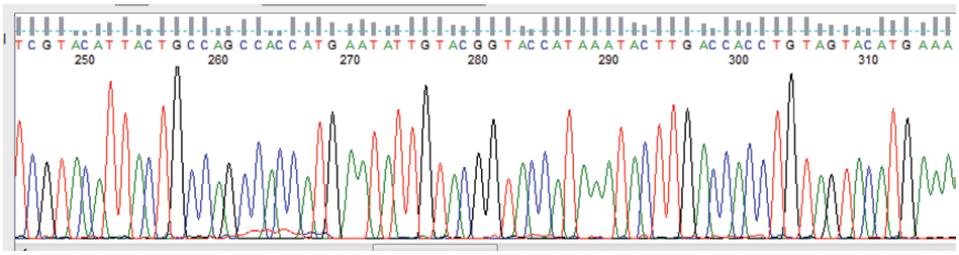

Figure 18.
Partial HVS1 sequence for DNA sample extracted from the burnt liver.

```
CLUSTAL format alignment by MAFFT (v7.452)


CRS             tcacgggagctctccatgcatttggtattttcgtctgggggggtatgcacgcga--tagca
1-1             ---------------------------------------------------------
1-5             ---------------------------------cgtctgggggggtgtgcacgcga--tagca
rel             ----------------------------------ctgggggggtgtgcacgcgaataagca


CRS             ttgcgagacgctggagccggagcaccctatgtcgcagtatctgtctttgattcctgcctc
1-1             ----------------------cccctatgtcgcagtatctgtctttgattcctgcctc
1-5             ttgcgagacgctggagccggagcaccctatgtcgcagtatctgtctttgattcctgcctc
rel             ttgcgagacgctggagccggagcaccctatgtcgcagtatctgtctttgattcctgcctc
                                  ************************************


CRS             atcctattatttatcgcacctacgttcaatattacaggcgaacatacttactaaagtgtg
1-1             ctcctattatttatcgcacctacgttcaatattacaggcgaacatacctactaaagtgtg
1-5             atcctattatttatcgcacctacgttcaatattacaggcgaacatacctactaaagtgtg
rel             atcctattatttatcgcacctacgttcaatattacaggcgaacatacttactaaagtgtg
                 *********************************************.************


CRS             ttaattaattaatgcttgtaggacataataataacaattgaatgtctgcacagccacttt
1-1             ttaattaattaatgcttgtaggacataataataacaattgaatgtctgcacagccgcttt
1-5             ttaattaattaatgcttgtaggacataataataacaattgaatgtctgcacagccgcttt
rel             ttaattaattaatgcttgtaggacataataataacaattgaatgtctgcacagccgcttt
                ************************************************.****


CRS             ccacacagacatcataacaaaaaatttccaccaaaccccccct-ccccgcttctggcca
1-1             ccacacagacatcataacaaaaaatttccaccaaaccccccctccccccgcttctggcca
1-5             ccacacagacatcataacaaaaaatttccaccaaaccccccctccccccgcttctggcca
rel             ccacacagacaccataacaaaaaatttccaccaaaccccccctccccccgcttctggcca
                ***********.****************************** ***************


CRS             cagcacttaaacacatctctgccaaaccccaaaaacaaagaaccctaacaccagcctaac
1-1             cagcacttaaacacatctctgccaaaccccaaaaacaaagaaccctaacaccagcctaac
1-5             cagcacttaaacacatctctgccaaaccccaaaaacaaagaaccctaacaccagcctaac
rel             cagcacttaaacacatctctgccaaaccccaaaaacaaagaaccctaacaccagcctaac
                ************************************************************


CRS             cagatttcaaattttatctttt----------------------
1-1             cagatttcaaattttatcttttggcggtatgcacttttaacaga
1-5             cagatttcaaattttatcttttggcggtatgcacttttaacaga
rel             cagatttcaaattttatcttttggcggtatgcacttttaacaga
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Figure 19.
mtDNA HVS1 sequences alignment in DNA samples extracted from burnt liver fragment (1–1), long bone (1–5) and victim's sister buccal swab (rel) (by MAFFT program (https://mafft.cbrc.jp/) compared to the Cambridge reference sequence (CRS)).

Both autosomal and Y-STR loci profiles were determined using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit and AmpFlSTR® Yfiler ™ PCR Amplification Kit (Applied Biosystems, USA). At the same time, the profile of autosomal DNA and Y-chromosome DNA were able to be successfully established in the objects removed from the fire pit, which completely coincided with the profiles

obtained in the material of the lower extremities, burnt liver fragments (**Figure 16**), burnt finger phalange (**Figure 17**) and the victim's clothes.

According to the preliminary investigation, the victim had a sister so in order to complete human identification, mtDNA analysis was carried out. Hypervariable regions, HVS-1 and HVS-2, of mtDNA D-loop sequencing was performed by the MitoPlex system (Gordiz, Russia). Individual sequences were also determined in the DNA samples extracted from lower limbs, burnt liver fragments and clothing items of the missing man, as well as from the buccal swabs of the woman believed to be the sister of the deceased man (**Figures 18** and **19**).

Thus, the testing that was conducted made it possible not only to identify the deceased man but also to prove the circumstances of the crime committed. This assisted the process of gauging the circumstances of the offending and the seriousness of the crime committed.

4. Conclusion

Based on our data, when analyzing highly degraded DNA, it is preferable to use information from all types of DNA markers such as autosomal STRs, X- and Y-STRs and mitochondrial hypervariable region sequence to get the best possibleindividual DNA profile, especially in difficult cases.

In the near future, in order to obtain the fullest possible individual DNA profile, it will be useful to include information about phenotyping features (such as skin, hair or eye color, skeletal particularities etc.) and biological age based on microarray analysis, next-generation sequencing SNP data, state of methylation and specific gene expression analysis.

But the highest percentage of reliable and reproducible DNA profiles can be obtained from the odontoid process of the 2nd cervical vertebra, long tubular bones of the lower (femoral) and upper (shoulder) limbs, as well as from teeth. In addition, even single hairs and burnt tissues can constitute a source for successful DNA identification.

The results of our analysis made it possible to formulate a number of recommendations regarding the collection of human remains from the crime scene in order to enable successful DNA analysis.

If a complete skeleton or unidentified corpse is found:

- in the absence of deeply penetrated putrefactive changes, it is advisable to provide bones with remnants of soft tissues, not only their single fragments;
- it makes sense to provide, first of all, ribs and long tubular bones of the lower and upper limbs as well as 2nd cervical vertebrae;
- it is advisable to provide several bones from one body, such as a rib, long bone, or a tooth;
- if an incomplete skeleton or individual bones are found, it is advisable to provide all the discovered bone remains, so that the forensic geneticist can independently decide which of the bones to examine first;
- if a body is found after a fire, it is good practice to provide tissue fragments (liver, blood vessels, etc.) for DNA identification.

When working at the crime scene special attention should be paid to searching and removal of small bones and fragments to make it possible to obtain complete genetic profiles. However, it is necessary to understand that not all objects allow the preservation of DNA for a long time [13] and bone remains are one of the most controversial objects. On the one hand, the physiological structure of the bone allows DNA preservation for a long time, but on the other hand, it is very difficult to extract DNA from some types of bones.

Besides, in the process of DNA extraction, a sample can be severely damaged or even destroyed, and as a result a false-positive result can be obtained (such as by contamination by other DNA samples) or a false-negative result (such as by the presence of inhibitors). That is why it is very important to eliminate the errors due to the reproducibility in DNA analysis.

Moreover, probability estimation in identification analysis also plays an important role in DNA analysis interpretation. Based on our experience, the Bayesian probability gives the most reliable conclusion about the non-random coincidence of determining genetic profile in case of positive individual identification [14]. In addition, it should be taken into account that for working with degraded DNA samples qualifications and experience of a geneticist should be confirmed with special competence documents.

Finally, it is important that the results of DNA identification should not be the only prosecution evidence. Where there are discrepancies with the materials of the investigation, it is necessary for further expert studies to be undertaken which take into account all the circumstances of the crime that has been committed [15, 16].

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

Probabilistic Genotyping: A Possible New Legal Avenue to Prevent and Redress Miscarriages of Justice

Geert-Jan Alexander Knoops

Abstract

This chapter delves into the relatively new DNA technique of probabilistic genotyping, which aims to a more precise determination of complex DNA profiles of multiple contributors. It explains the forensic value of this methodology compared to traditional DNA techniques such as Combined Probability of Inclusion (CPI). In particular, this forensic value is demonstrated in light of the reversal of several wrongful convictions in the USA and Europe. Apart from having a potential exculpatory effect, the advance of probabilistic genotyping can also contribute to discerning the real perpetrator of a crime. As a result, this chapter emphasizes the relevance of probabilistic genotyping for both defense lawyers and prosecutors in criminal cases.

Keywords: Mark Perlin, Greg Hampikian, DNA evidence, forensic evidence, probabilistic genotyping, DNA mixtures, wrongful convictions, defense lawyers, prosecutors, Lydell Grant, Dutch Proveniers case

1. Introduction

This chapter discerns the implications for law practitioners of the DNA technique of probabilistic genotyping. This method was developed in the late 1990s. It uses statistical methods and mathematical algorithms in DNA profiling, instead of applying manual methods to determine very small DNA samples or DNA mixtures of multiple individuals, and it calculates likelihood ratios while inferring genotypes of a DNA profile based on computer software, "Probabilistic Genotyping Software (PGS)", models by intricately unraveling all parts of the mixture. Before addressing the implications, one should first look at the limitations of current DNA techniques.

In their article of March 2014, Perlin et al. stated that "DNA analysis is the gold standard of human identification" [1]. This observation can also be found in other academic publications such as the 2011 article of Dror and Hampikian [2]. The latter experts note that "DNA has been held as objective and immune to subjectivity and bias". However, they add that "(…) at the least in complex situations (such as with DNA mixtures) DNA does require and rely on human examiners making a variety of subjective judgements, that are susceptible to bias (…)". Thus contrary to what one may expect from a gold standard of forensic science, DNA cases pertaining to complex mixed profiles may create subjectivity and trigger contextual bias.

1.1 The current pitfalls of DNA evidence for criminal cases

Dror et al. demonstrated this phenomenon with an experiment, using a DNA mixture analysis from a real criminal case. Their analysis was presented to seventeen independent DNA experts in the USA, without the potentially biasing contextual case information. The test was to examine the DNA mixture along with DNA profiles of the victim and three suspects. The focus was suspect three. This suspect was labeled by the DNA experts who were assigned in the real criminal case as "cannot be excluded." These experts were given the actual contextual potential biasing information. The seventeen experts in the test were only provided the sperm fraction electropherograms from the victim's vaginal swab after amplification with cofiler (ABI) and the DNA concentration in the sperm fraction extract and injection times and were asked to give one of the following three conclusions: "cannot be excluded," "excluded" or "inconclusive." The outcome of this test was quite revealing: one expert arrived at the conclusion that suspect three "cannot be excluded," while four experts held the analysis to be "inconclusive" and twelve experts determined this to "exclude" suspect three. These differences are especially striking in light of the fact that these seventeen experts all worked in the same accredited government laboratory and applied the same interpretation guidelines.

Two conclusions can be derived from this experiment. First, there is an element of subjectivity in the assessment of DNA evidence by—even qualified—DNA experts who even used the "golden DNA standard" and identical evidence. If total objectivity would have existed, all the experts should have arrived at same conclusion, because the experts work at the same laboratory and use the same guidelines. Second, there was a pertinent difference between the assessment made by the DNA experts in this experiment who had limited contextual information of the criminal case and the original experts who had access to the biased context of the criminal case. The experiment of Dror et al. shows that only one out of seventeen experts arrived at the same conclusion as the original experts, while sixteen other experts came to a different and conflicting conclusion. The conclusion of this experiment study is that "(...) the extraneous context appears to have influenced the interpretation of the DNA mixture (...)" [2].

This study illustrates that when it concerns DNA mixture analysis, the "golden standard" qualification must be nuanced in that DNA mixture interpretation inheres subjective elements and is exposed to bias and even contextual influences. It is therefore of paramount importance that prosecutors, defense counsels and trial judges are aware of these potential subjective influences when confronted with criminal cases where low numbers of template molecules are amplified or where complex mixtures are examined. Notably, quantitative conclusions as "cannot exclude" are mostly presented by forensic experts without quantitative measuring [2]. To remedy this pitfall, the 2010 scientific working group on DNA analysis methods (SWGDAM) in Section 4.1 of their guidelines promulgated that "the laboratory must perform statistical analysis in support of any inclusion that is determined to be relevant in the context of the case, irrespective of the number of alleles detected and the quantitative value of the statistical analysis" [2]. The International Society for Forensic Genetics (ISFG) also endorses the same approach in respect to the interpretation of mixtures [2]. However, scientific research also shows the inclusion of statistical analysis in support of certain DNA conclusions and does not remedy the element of subjectivity and potential bias [2]. After having observed that the "golden standard" of DNA evidence is less "golden" and might be "silver," the question arises as to the implications thereof for the criminal law practice.

1.2 The importance of erasing contextual bias: the Lydell Grant case

"To error is human, to correct error is responsible science." These are words of Greg Hampikian, Professor in Biology and Criminal Justice at Boise State University [3], which trigger the question whether and how criminal law practitioners might be able to correct such errors. This question first refers to the moral-ethical perception as to the functioning of the system of a criminal law in our society and second it refers to which legal avenues are available to remedy such errors.

One may illustrate this on the basis of the case of Lydell Grant, who was convicted of murder in a 2010 stabbing, which resulted in the killing of a 28-year-old man outside a Houston (USA) nightclub, in Montrose District, the center of Houston gay cultural life, and sentenced to life imprisonment. In November 2019, the Harris County District Attorney's office ordered the 42-year-old Lydell Grant be released on bond, pending a reinvestigation of the case. The events leading up to this decision were remarkable. The night after the assault, the barback of the nightclub spotted a tall, masculine black man with short hair, stepping out of a white Pontiac Grand Prix. The man entered a different nightclub. The barback seemed to recognize the assailant of the previous night. He wrote down the license plate number of the Pontiac and tipped the Houston Police. The license plate number belonged to Lydell Grant, at that time 33 years old, who had a criminal record. The Houston Police Department therefore had access to his photo. Based upon a photo spread including Grant's photo and five other young black men, six eyewitnesses, who at the time of the attack watched the whole incident unfold in just a few minutes from the second floor patio of the nightclub, identified Grant as the person who killed the victim, who was named Aaron Scheerhoorn.

The prosecution's evidence against Grant thus relied on six eyewitnesses who testified at trial with high degrees of certainty that Grant stabbed Scheerhoorn. Out of these six, three testified that they were "positive," two stated they were "one hundred percent sure" and another witness was "very sure," saying that Grant's face "was burned into my memory immediately" [4]. The relevance of the Grant case for the topic of this chapter relates to the rule of DNA evidence. Apart from the six eyewitnesses, the prosecution's case against Lydell Grant was built upon DNA analysis conducted by a DNA expert from the Houston Police Department's crime lab. This analysis had retrieved DNA profiles of two individuals under Scheerhoorn's fingernails. However, this expert was only able to detect a full profile of one of them, which according to this expert, belonged to the victim [4]. At trial, this expert provided "muddled" information as to her findings regarding the second profile [4]. Testifying that she could not exclude Grant, this expert therefore suggested that Grant's DNA was potentially to be found under Scheerhoorn's fingernails. Grant's defense counsel neither presented any contraexpertise to the jury nor challenged the DNA results, apart from obtaining the admission made by the expert at trial that Grant could not be "associated" with the DNA mixture [4].

Grant's defense counsel, in its defense case, called only one witness, Mr. Paul Rodriguez. This person testified that on the night of the assault in December 2010, Lydell Grant had been in his company the whole night, going from bar to bar without visiting the bar where Scheerhoorn was attacked, which was called "Club Blur." After three days of trial, the following day the jury only needed a four-hour deliberation: Grant was found guilty and sent to prison for life. Thereafter Grant started the "Herculaneum" endeavor to proof his "factual innocence" [4] within the prison where he stayed. He began to write dozens of letters to lawyers and several Innocence Projects in the USA, including the Innocence Project of Texas. Only few responded [4]. With his younger half-brother Alonzo Grant, he discussed his case, while spending every free minute in the library of the prison with an inmate he met

in the library. Grant went through the nine volumes of trial transcripts, searching for loopholes and inconsistencies in the witness statements [4]. It was at that time that his library inmate discovered that the DNA results that were seemingly "muddled" were actually to Grant's advantage. To his surprise, Grant read in the DNA expert report that "(...) no conclusions will be made regarding Lydell Grant as a possible contributor" [4].

It was at that moment that Lydell Grant realized that his defense counsel at trial had missed an opportunity to prove Grant's innocence, namely by not retaining an independent DNA expert to challenge the report of the Houston Police Department's crime lab. It took Grant till January 2018, 8 years after his conviction, and a turning down of his appeal in 2014, to find a defense counsel who was willing and able to file a motion to the court for a review of the DNA evidence ([4]: see Hall, o.c. at 10–11, who refers also that one lawyer was appointed to Grant in 2016 and wrote that he was "unsure of how DNA could help you"). It was also in 2019 that one of the letters Grant distributed arrived at the Executive Director of the Texas Innocence Project (IPTX), Mr. Mike Ware. Since 2006, when this nonprofit organization started, it achieved to exonerate 27 wrongly convicted inmates in Texas. Grant's case was accepted by this project and allocated to one of the students of the "Actual Innocence Clinic," which was part of the Texas A&M School of Law in Forth Worth. Research of the Innocence Project and the National Registry of Exonerations indicated that mistaken eyewitness identification is one of the most prominent causes of wrongful convictions, while discerning that people of one race have serious problems with identifying persons of another race [4]. This element that contributes to wrongful convictions features in some two-fifths of all exonerations based on DNA [4]. Interestingly, in the case of Lydell Grant, one of six eyewitnesses was a black person, two were persons of Latino descent, one was Asian-American and two were white people. Moreover, research reveals that showing witnesses a photo lineup of multiple photos such as in the Grant case could also result in mistaken eyewitness identification in that the witness identifies a person merely because that individual resembles the suspect more than anyone in the photo lineup [4]. At the time that Lydell Grant was arrested in 2010, the Texas legislature did not yet implement a law that required for "double blind" lineups. This means that the police officer conducting the lineup is not aware who the suspect is. Accordingly, he or she cannot influence the eyewitness by, for instance, making a comment during the lineup or making a certain gesture toward the witness [4]. The Texas Innocence Project detected several errors in the lineup procedure in Grant's case, while at the same time discovering that the DNA profiles in this case contained several alleles (i.e., the repeating genetic variations that result in the profile) and were not related to either the victim or to Lydell Grant [4].

In 2019, the Texas Innocence Project presented these findings to Dr. Angie Ambers, a forensic DNA expert and Associate Professor of Forensic Science at the University of New Haven in Connecticut and Assistant Director of the Henry C. Lee Institute of Forensic Science. Dr. Ambers converted the DNA data from the original test into an Excel spreadsheet, and based upon this review, she determined that 26 alleles in the mixture were not related to either Grant or the victim, but to someone else [4]. To confirm her analysis, in March 2019, the data were sent by Dr. Ambers and the IPTX to Cybergenetics Corporation in Pittsburgh. Cybergenetics is the developer of the leading software program TrueAllele, which is a new method to analyze DNA mixture with more precision, based on a software system, using statistical methods. This method, named "probabilistic genotyping," was developed in the late 1990s. It uses statistical methods and mathematical algorithms in DNA profiling, instead of applying manual methods to determine very small DNA samples or DNA mixtures of multiple individuals, and it calculates likelihood

ratios while inferring genotypes of a DNA profile based on computer software, "Probabilistic Genotyping Software (PGS)," models by intricately unraveling all parts of the mixture. It therefore advances the statistical analysis of DNA mixtures. Cybergenetics was not only able to exclude Lydell Grant but also was also able to deduct a second profile of an unknown DNA contributor. After deducting this profile, it was uploaded in the Combined DNA Index System (CoDIS) of the FBI. This additional database contains the data of approximately 14 million convicted people in the USA. It was in July 2019 that the FBI database connected the profile to a prisoner named Jermarico Carter, who had a criminal record in Houston and had moved to Atlanta a few months after the incident. The mistaken witness identification of Grant was to be explained by the fact that Carter—similar to Grant—was black, had a similar posture and was of the same age [4].

After Carter had been arrested in Atlanta on a parole visitation, he denied to be the perpetrator of the deadly stabbing of Scheerhoorn. However, when the police detectives told him that his DNA was found under the fingernails of the victim, Carter admitted that he had fought with Scheerhoorn and chased him to the nightclub where he, as he asserted, only hit him. Three months after Carter's confession, the defense for Grant filed a writ of habeas corpus based on "actual innocence," arguing that inaccurate DNA evidence and mistaken eyewitness identification had violated Grant's due process rights. The DNA results and Carter's statement led the Houston Police Department to reinvestigate the Scheerhoorn case. The results were astonishing: no link whatsoever was found between Grant and Scheerhoorn, while it was established that Carter had indeed lived in Houston in 2010 at the time of the crime [4]. Moreover, Carter was arrested in the Montreal District on another occasion. Four months after the Scheerhoorn incident, he was also arrested for stabbing a person in Atlanta. As a result of this new evidence, Lydell Grant was released from Harris County Prison based on a bail-bond of $ 100.000 USD. In the meantime, the investigation by the police and the office of the District Attorney continued, which in December 2019 resulted in the District Attorney's office dropping Grant's case and charging Carter for the Scheerhoorn case. At the same time, the Houston Police Chief issued a public apology to Lydell Grant and his family [4]. One week later, the trial judge accepted the habeas corpus writ filed by Grant's defense counsel and held that he proved to be actual innocent [4]. However, with writs, as opposed to direct appeals, when both the prosecution and the district court agree on the actual innocence, this solely results in a recommendation for the Texas Court of Criminal Appeal (CCA), which has the final say in exonerations. In April 2020, the CCA overturned this decision of the district court and requested additional evidence, namely Carter's confession tape. This was very unusual, but the Texas Innocence Project complied and waited. Then, on July 1st, the CCA first ordered the case to be remanded back to the District Court, second that the District Court ask the DA's office to get the six eyewitnesses' accounts at Grant's trial to respond to Grant's innocence claims and third that the District Court provide the CCA with a photo of Jermarico Carter, dated from the approximate time of the crime [5]. In fact, the CCA—comprised of nine judges—all elected—conveyed the message that the presented evidence did not yet establish Grant's innocence.

Experts, former CCA judges, prosecutors and also Dr. Ambers, the DNA scientist who was instrumental in exonerating Lydell Grant, were puzzled with the CCA ruling. Dr. Ambers commented that she does "(...) not know what else Grant could do—it doesn't get any more definitive than that" [4]. The Grant case is but one example of many, whereby the methodology of probabilistic genotyping was decisive in the last decade to exonerate a convicted person. In May 2020, the Boise State Laboratory (USA) led by Professor Greg Hampikian was able to exonerate Johnnie

Lee Gates, a Georgian man who turned out to be wrongly convicted of rape, armed robbery and murder in 1977, based upon a new DNA review using probabilistic genotyping [6]. The advent of probabilistic genotyping has therefore contributed considerably to establishing actual innocence within the USA [7].

1.3 Differentiating common DNA methods from probabilistic genotyping as a means to prevent and redress miscarriages of justice

After having determined that several exonerations in criminal cases were predominantly fueled by advancing probabilistic genotyping, the question arises whether empirical evidence exists to the extent that common DNA methods can wrongly include innocent individuals as contributors to mixed DNA profiles as opposed to probabilistic genotyping. The question can be answered in the affirmative. In 2019, the National Institute of Standards and Technology (NIST) demonstrated that common DNA methods such as the Combined Probability of Inclusion (CPI) have wrongly included innocent persons as being contributors to DNA mixtures [3]. Three years prior, in 2016, the President's Counsel of Advisor's on Science and Technology issued a report that concluded that "In summon, the interpretation of complex DNA mixtures with the CPI statistics has been an inadequately specific—and thus inappropriately subjective—method. As such, the method is clearly not foundationally valid" [8]. Hampikian describes an interlaboratory study based upon a fictional scenario, conducted by Butler [9]. Several North American Forensic DNA labs were asked to analyze a ski mask from a bank robbery with a complex DNA mixture of at least three contributors, suspects A, B and C. The main question was whether the specific laboratory deemed the mixture as too complex to make any findings. The second test was to determine if one of the labs wrongly included a person in this mixture (false positives). The results were similarly striking. Out of the 108 accredited labs, 68% of them wrongly included suspects C, who was—for the purposes of this test—innocent. As to the question whether the DNA mixture was too complex to draw conclusions from it, 27 (25%) of the labs held the mixture to be inconclusive. Only seven labs (6%) correctly excluded the suspect. However, notably 74 of the 108 labs (68%) included an innocent person based on a match statistic. Professor Hampikian qualifies these results as a "chilling conclusion" [3]. He also refers to the "Georgia case" study, mentioned in paragraph 1, to conclude that "(...) the good news is that the only lab that used probabilistic genotyping software (TrueAllele by Cybergenetics) in the NIST study, got the right answer and excluded suspect C. To sustain this conclusion, Hampikian mentions the Virginia study of 2014 by Perlin in which 144 old cases, which were based on CPI methodology, were reexamined on the basis of the TrueAllele program [3]. In five of these cases, the TrueAllele program excluded the profile that was included by the manual CPI method. As a result, Hampikian concludes that probabilistic genotyping can often tell if their claims (convicts who claim to be innocent) are true [10]. The next paragraph will delve into the question as to the implications for the criminal law practice, that is, for defense counsels and prosecutors in criminal cases.

2. Implications for the criminal law practice

2.1 Introduction

The advent of probabilistic genotyping began in 1999, when Mark Perlin of Cybergenetics Corporation in Pittsburgh (USA) developed the program "TrueAllele." Since then, it has served criminal cases both to the benefit of defense and also to the interest of the prosecution.

2.2 Probabilistic genotyping as a defense tool

For the defense, probabilistic genotyping has demonstrated its relevance in overturning wrongful convictions. The first use of probabilistic genotyping to redress a wrongful conviction was the exoneration of Darryl Pinkins and Roosevelt Glenn in 2016. These persons were convicted of rape and robbery in 1990 and were sentenced to 65 years imprisonment. The case against Pinkins was built upon an eyewitness identification by the victim as being one of the assailants and a statement from an inmate who shared a prison cell with him, alleging that Pinkins had confessed the rape to him. The case against Pinkins lacked forensic evidence, more specifically DNA evidence. After the conviction, the DNA traces from unknown persons found on the victim's clothes were examined with probabilistic genotyping. The result was that none of the five DNA traces found on the victim's clothing matched with Pinkins' DNA [11]. The 2016 Pinkins case was the first example whereby the TrueAllele software analysis developed by Dr. Mark Perlin was able to completely exclude the convicted person Darryl Pinkins and Roosevelt Glenn from the semen evidence that was a mixed profile stemming from a multiple perpetrator sexual assault case. After 23 years in prison, both persons were exonerated [12].

Soon thereafter, the defense of Mr. Johnny Lee Gates was able to achieve a similar exoneration based on probabilistic genotyping. Gates was wrongly convicted in 1977 for rape, armed robbery and murder. He was found guilty of shooting and killing a 19-year-old victim in her apartment where she lived with her husband. Also here, probabilistic genotyping showed that Gates' DNA was not to be found on crucial pieces of evidence. In particular, it was not detected on the white belt from the victim's bathrobe and black neckties that was used by the perpetrator to blindfold the victim. Had Gates been the perpetrator, his DNA had to be left on these items [4]. The method of probabilistic genotyping ensured that Johnny Lee Gates after 40 years of imprisonment was released from prison and found innocent.

Following the Gates case, more exonerations emerged in the USA based on probabilistic genotyping. Mention is made of the exonerations of Freddy Lawrence and Paul Jenkins who were wrongly detained for 23 years. They were convicted of robbing, abducting and eventually killing a 34-year-old Donna Meagher in the Jackson Creek Saloon in Montana City in 1994. The crime went unsolved for some time, until Lawrence's father-in-law Dan Knipschield stated that his son-in-law was involved in the crime. The police then asked him to wear a tape recorder to record a confession. The tape recorder malfunctioned, and no recording was made. Nonetheless, Knipschield told the police that Lawrence confessed to having committed the crime along with Paul Jenkins. As a result of this, Lawrence was questioned by the Montana Police Department. That's when Lawrence stated that he had no involvement in the crime, but Jenkins and Jimmy Lee Amos, a mentally challenged man who lived with Jenkins and his wife, did. Lawrence later recanted this statement, but the police officers interviewed Jenkins' wife Mary, as well as Amos, anyway. Mary made an incriminating statement, but she also had a severely diminished mental capacity. There was no recording of Mary's interrogation, although she was interrogated for 8 hours straight. Amos was declared incompetent to testifying because of his diminished mental capacity, but Mary's statement was included at trial. Lawrence and Jenkins were convicted by separate juries and sentenced to 100 years in prison, merely on the basis of two statements, of which there were no recordings. There was no physical evidence linking either man to the crime at all. In 2015, the Montana Innocent Project filed a motion seeking DNA testing of the physical evidence. Meanwhile, Fred Nelson came forward, who said that his uncle, David Nelson, had admitted to having committed the crime. He could also provide details about the crime that matched the police's details of

the crime. Fred Nelson had already told the law enforcement about this a couple of months after the crime, but the police told him nothing could be done because there was no evidence. In 2018, the Montana Innocent Project filed a motion to vacate the convictions. Also in this case, probabilistic genotyping proved that the DNA evidence did not match either Lawrence or Jenkins. However, the DNA profile recovered from a piece of rope found near Meagher's body did match David Nelson, whose DNA profile was already stored in the Montana State DNA database. District Court Judge Kathy Seeley granted the motion in April 2018 [13]. The exonerations demonstrate that it is of perennial importance that defense counsel representing defendants who are charged based upon DNA inclusion pertaining to complex DNA mixtures of multiple persons or perpetrators endorse probabilistic genotyping.

In other countries probabilistic genotyping could serve as a more precise alternative for the traditional DNA methods as well. Another example of a possible wrongful conviction based on erroneous DNA evidence emerged in the Netherlands, where real estate broker Victor 't Hooft was shot and killed on the 7th of November 2007. There was only one witness in this case, Mr. 't Hooft's wife, Mrs. Emmy van Dijk. She testified that the killer rang the doorbell, and when she opened the door, there was an intruder, who was described as a white male, around 1.90 meter tall. Immediately, a fight between Mr. 't Hooft and the shooter began, and Mr. 't Hooft was shot right away. Mrs. van Dijk had testified there being a moment when she and the shooter were alone in a room. Yet, no injuries were found on her. The defendant Remond Proveniers' DNA was found on the gun and on the casings of some of the bullets that were found at the crime scene. Remarkably, not all the bullet casings that were found at the crime scene were originating from the gun that was found. Consequently, there must have been a second gun, being (one of the) the murder weapon(s). The court ruled that the DNA evidence did not match the other evidence, and therefore he was acquitted in first instance. However, in appeal, the court came to a different evaluation of the DNA evidence and Mr. Proveniers was convicted purely based on the DNA evidence [14]. The defendant provided two explanations for the DNA on the gun, which should have exonerated him. First, he stated that a few months prior to the shooting, his gun, gloves and some other munitions were stolen from his safe and these items could have been used for the shooting. That way, the real perpetrator would have had access to his gun and Proveniers' DNA would also be on the gun. The second explanation pertained to the fact that Proveniers had been to a gas station prior to the crime. He had sneezed over the cashier desk and therefore his DNA would be on the cashier desk. The video cameras from the gas station displayed that Mr. 't Hooft's wife, Mrs. Van Dijk, arrived at the same gas station's cashier desk about 7 to 9 minutes later than Mr. Proveniers. Proveniers states that because he and Mrs. Van Dijk have stood behind and touched the same cashier desk, his DNA could have been on Mrs. Van Dijk's hands, and therefore it could be possible that the DNA was found at the crime scene. Besides, Proveniers is a so called "strong shredder," meaning that his DNA transfers to other surfaces really easily in comparison to other people. The only evidence linking Proveniers to the case is his DNA evidence, while the two possible explanations of Mr. Proveniers were not sufficiently ruled out. New DNA methods, such as probabilistic genotyping, could assist in determining this potential miscarriage of justice. This approach was also affirmed by Dr. Greg Hampikian, who has drafted an affidavit in 2020 recommending further analysis of the DNA evidence in this case with the probabilistic genotyping method.

The Proveniers case reveals some other pitfalls in regard to (traditional) DNA methods being considered the "golden standard" of evidence. First of all, there's a possibility of secondary transferring. Proveniers' explanation of "the sneeze on the

cashier desk" remains forensically possible. If someone's DNA is found on a crime scene, it does not automatically mean that the person was the perpetrator, or even that the person was present at the crime scene. DNA can be easily transferred from object A to object B or from person A to person B. The second issue relates to the first, in the sense that it is possible that DNA evidence can be contaminated, meaning that DNA traces from the crime scene can be inadvertently mixed with DNA of third persons (e.g., by not properly storing), who have no relation to the specific crime [15]. That way, DNA samples can be retrieved from the evidence, without the donor being the perpetrator. These limitations illustrate that not only at the stage of judging the evidence mistakes are made and caution is advised, but also in the stage of retrieving evidence from the crime scene.

Yet, a critical remark about probabilistic genotyping is to be made. According to Richard Torres, a staff attorney in the DNA unit of the New York Legal Aid Society in New York City, and scientists such as Dan Krane, who is a professor of biological sciences at the Wright State University, there is a problem with the lack of transparency. Organizations like Cybergenetics provide the tool and supplemental materials such as validation studies to defense counsels, but the provided information is incomplete to protect the company's intellectual property. Torres argues that the defense had a right to confront and question the algorithm, not just the scientist who made it [16]. To remedy this potential problem, the defense could seek for a court order to disclose the algorithms and the underlying source codes in order to verify this information and to question the forensic expert about the use of these source codes. Actually, the defense should have access to all source materials relating to DNA methods. The study of Thomson et al. [17] already indicated the importance of the defense having full access to the underlying source materials, which can not only reveal forensic errors but also DNA traces of unknown persons. **Figure 1** displays electropherograms from a rape and homicide case. In that case, the defendant admitted having intercourse with the victim, but the defendant also said that another man had raped and killed the victim afterwards. The crime lab only reported the defendant's DNA profile in the vaginal samples from the victim, but a review of the electronic data by a defense expert revealed low-level alleles

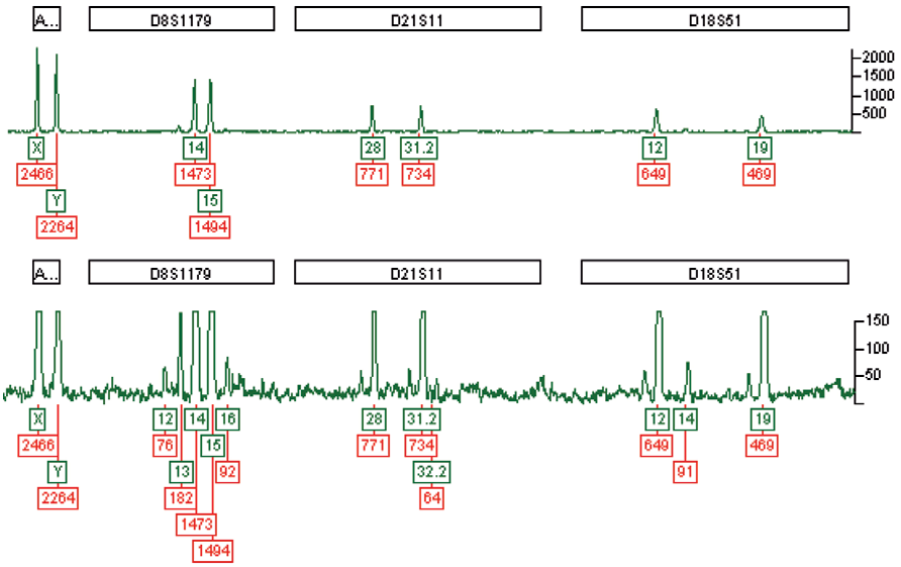

Figure 1.
Defense examination of electronic data [17].

consistent with those of the second man, which contributed to the exoneration of the defendant. The low peaks are revealed in the lower electropherogram, where the defense expert set the software with a lower threshold of detection and produced an electropherogram with a lower scale. Even though this case does not relate to probabilistic genotyping, it is a clear illustration of the importance of the defense having access to all the source code information [17].

Finally, while probabilistic genotyping can be instrumental in unraveling a cold case, it has to be stressed that a DNA profile as such, without corroborating evidence, should never be the sole piece of evidence. In a criminal case, it simply does not tell the judge who is the real perpetrator. DNA evidence therefore should be just one part of the evidence in a criminal trial. However, as indicated by Dr. Mountain "A DNA profile is rarely the sole piece of evidence; it is not allowed to be in the UK" [18]. However, probabilistic genotyping should be admissible as evidence when it serves an exculpatory purpose, when it that is excludes the defendant from the crime.

2.3 Probabilistic genotyping as a prosecutorial tool

Contemporary criminal law practice also reveals that probabilistic genotyping can assist law enforcement officers in solving cold cases as well as assist in pending prosecutions. An illustration thereof is the Syracuse case. The case related to Frank Thomas who had pleaded not guilty to charges pertaining to illegal possession of a weapon, reckless endangerment and threatening a police officer. In 2014, on the 21st of August, two Syracuse police officers tried to stop a car driving without headlights. The driver and the passenger fled and fired two gunshots at the police officers. The officers were unable to find the car that night, but they did find the gun with which the shots were fired. On the gun, five different DNA samples were found. The mixture of DNA on the gun was too complex to analyze with traditional DNA methods, so the prosecution endorsed the application of TrueAllele software analysis, which ultimately showed that one of the five DNA samples did match Frank Thomas. Thomas was ultimately found guilty of criminal possession of a weapon, reckless endangerment and menacing a police officer. He was sentenced to $15\frac{1}{2}$ years in prison [19].

One has to bear in mind that during the last decade, the number of cold cases has increased. In the Kingdom of the Netherlands alone, in 2019, around 1500 severe criminal cases remained unsolved [20, 21]. According to Project Cold Case, a non-profit organization with the goal to help solve cold cases, nearly 185,000 cases of homicide and nonnegligent manslaughter went unsolved from 1980 to 2008, a total that still increases every year [22]. A more systematic application of probabilistic genotyping on cold cases could contribute to the unraveling of perpetrators who otherwise might remain forensically undetected.

3. Conclusion

This chapter first has outlined some of the limitations of traditional DNA methods within the contemporary field of forensic evidence. Moreover, this chapter has illustrated the danger of a lack of objectivity when it comes to evaluating DNA evidence. Research suggests that contextual information of the crime especially can lead to subjective outcomes, which can result in experts in the field having different opinions about the same DNA sample. One can imagine that this might have a damaging effect on the legal system. One of the reasons for this is that DNA evidence

is still seen as the "golden standard" of all evidence by law enforcement officers. However, it has been shown that one DNA sample can lead to different conclusions about who the donor is. As a result, the "golden standard" rule should be nuanced. At the least, it is to be recommended to always seek for a second opinion as a standard when using the traditional DNA methods. As an alternative for the traditional DNA research methods, this chapter also determined the effects of a relatively new method named probabilistic genotyping for the criminal law practice, while foreshadowing its relevance for, in particular, the redressing of wrongful convictions as well as resolving cold cases. As such, the TrueAllele software program advances an important goal in the truth-seeking nature of criminal law, namely the exclusion of subjectivity and (contextual) bias in the identification of the real perpetrator of crime, while excluding the innocent.

This chapter arrives at the following conclusions. First, for defense counsel in criminal cases, it is to be advised that particularly in cases involving DNA mixtures entailing profiles of two or more profiles of individuals other than the victim's profile, recourse to probabilistic genotyping can be a forensically powerful instrument. This is specifically relevant when the DNA results based on the traditional methods are not conclusive or, even, when the DNA of the defendant is found in the mixture, while the defendant nonetheless claims to be innocent. Secondly, the studies mentioned in this chapter demonstrate that the interpretation of complex DNA profiles by forensic experts is not hard science, that is, susceptible to a certain level of subjectivity. Not only the defense lawyers but also prosecutors and the judiciary should be conscious about the phenomenon. By timely acknowledging these potential DNA evidentiary pitfalls, one can prevent miscarriages of justice. Specifically, it implicates that the precise context information the DNA expert is given for this assessment should be disclosed in order to ensure that both the inculpatory but also the exculpatory context information was made available to the experts. The disclosure therefore might be decisive for the judicial appraisal of the DNA evidence. Thirdly, in criminal cases in which the prosecution relies on DNA evidence based on traditional methods, it is important that defense lawyers have access to all source materials, as well as call for contraexpertise. The case of Lydell Grant demonstrates that a review of DNA evidence by a second expert might prevent wrongful convictions.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

Forensic Analysis and Interpretation of Tool Marks

Sachil Kumar, Geetika Saxena and Archana Gautam

Abstract

The forensic analysis and interpretation of tool marks raise for consideration key methods and advances in the field of tool marks in forensic science. This chapter shows how tool mark analysis can be utilized in the course of criminal investigations. The focus of the chapter is on bringing together as much scientific knowledge in the area as possible in an accessible manner. It covers all aspects of tool mark evidence from the crime scene to the courtroom. This chapter provides information about tool marks in an effort to assist tool mark examiners as well as people practicing forensic science, crime scene examiners, crime investigating officers and members of the legal profession. It includes information about the analysis of tool marks at the crime scene and in the laboratory, the interpretation and assessment of challenges for examination and interpretation and also the way in which tool mark evidence can be presented in a courtroom.

Keywords: forensic analysis, tool marks, investigation, court, crime scene, interpretation

1. Introduction

Tool mark identification is a fascinating forensic science discipline. By comparing the pattern of the tool marks in question and the pattern of the tool marks generated by the tool in a laboratory environment, a skilled analyst can give an opinion based on the accuracy of the questioned tool mark produced by a specific item [1]. This assists the forensic investigator in matching the marks on tools to crime scenes. Forensic tool mark identification includes firearms identification, an area of tool mark investigation that specializes in identifying different firearms and parts of a firearm being used at crime scenes. It also includes fracture matching or a physical fit [2], whereby two specific objects are analyzed to determine whether they have been at one time a single unit. If that is the case, the investigator will further analyze how the two objects come into contact and how they affect each other.

Tool marks can be generally understood as impressions or marks that are produced by a tool [3]. When a tool contacts a surface with sufficient force, a mark or an indentation is permanently left on the receptive surface.

A striation, as defined by AFTE, is a range of marks on the surface of an object [4]. These marks are produced by a combination of impact and motion. A pry mark made by the tip of screwdriver is a type of striated tool mark [2]. Similarly, an impression can be defined as a range of marks on the surface of an object [4]. As with a striation, an impression is produced by a mixture of impact and motion.

Notably, impressions are not caused by strong impact but appear on a surface as soft or shallow indentations. A hammer impact is a type of impressed tool mark.

Tools may be connected to tool marks and vice versa due to certain patterns or anomalies during the manufacturing process embedded in their surface. It is argued that patterns and anomalies of the tool mark are specific to each tool; the distinguishing features of a particular tool may be one aspect, just as the markings on a bullet can lead to a particular one and can be identified and compared visually. In consideration of this, a forensic investigator can become familiar with the manufacturing processes used to manufacture the working surface [5] of a tool and can compare the class features with the same surface of the tool such that it is possible to measure the uniqueness of a tool and its tool mark. Knowledge and understanding of tool manufacturing methods, along with close examination of tools and markings of tools, will make it easier to carry out this particular recognition.

There was no direct way in the past to associate a tool mark with the tool itself, and little progress has been achieved with the advent of modern forensic technology. In using tools to gain entry, a burglar will invariably leave tool marks behind that are of forensic significance and potentially incriminating, which can provide vital evidence to investigators and prosecutors. Given this, the essential factors that influence both tool mark production and the subsequent inspection of such marks in the forensic examination can be determined. These factors include the following:

1. The surface material that the tool is functioning on
2. The material used in order to construct the tool
3. The relative hardness of each material
4. The manufacturing procedure followed in order to construct the tool
5. The tool operational surface [6].

2. History of tool marks

Since many previous centuries, a historical understanding of the tool mark has been recognized that marks can be connected directly to tools, but few written references are typically found on this specific subject. A cited example often comes from China in the Twelfth century, where various wound shapes created by cutting tools such as sickles were considered, but even in China, there is little evidence of their importance.

Henry Goddard (1800–1883) of Scotland Yard is remembered as the first investigating officer to collect forensic evidence by analyzing a bullet and its related pattern to investigate a murder [7]. In 1835, using a bullet recovered from the autopsy victim's body, a defect was discovered that could be traced back to the original mold from which the bullet was made. In 1891, Hans Gross published a book entitled "Handbuch für Untersuchungsrichter als System der Kriminalistik" detailing all the basic precautions for the analysis of tool marks [8].

In 1953, a popular book entitled "Crime Investigation" textbook written by the renowned criminalist, Paul Leland Kirk (May 9th, 1902 – June 5th, 1970), explains the need for cast marks found in crime scenes if the item with the mark cannot be transported to the laboratories and makes a strong distinction between "compression marks" and "sliding marks." In his book, he examined immersed marks by using macrography while comparison microscope was used to analyze striated

marks, along with the examination of physical fit. In the 1974 edition, there is a reference to the work by Biasotti [9], *The Principles of Evidence Evaluation" as applied to Firearms and Tool Mark Identification*, which contains some of the first references for objective methods for evaluating striated marks.

In 1958, a book entitled 'An Introduction to Tool Labels, Weapons and the Striagraph' was written by John E. Davis, a prominent criminalist and the chief of the Oakland Police Department (CA) Criminalistics Division (Crime Lab). This textbook also introduced a new advanced piece of research equipment called "Striagraph," which was able to calculate, trace and record microsurface contours and was the precursor to advanced laser and digital imaging techniques for future bullet surface scanning technology [10].

The Association of Firearms and Tool Mark Examiners (AFTE), an international nonprofit organization devoted to facilitating the identification of firearms and tool marks, was founded in the United States in 1969 [11].

3. Definition of tool marks

Tools are mostly directly related to object markings, because at the time of tool production, such designs or irregularities are imprinted on their surface, so it is implied that these patterns and variations might be part of the identification features of a particular object; for example, marking bullets can lead to a particular firearm. Furthermore, these substantially different types and irregularities of the instrument can be visually identified and compared using forensic techniques [12].

The term "tool mark" is defined in a number of ways. A widely accepted AFTE definition defines tool mark as "If any object or instrument reaches the surface with enough force to allow its signature design to be indented, this form of marking is referred to as a tool mark.". In another definition [6], it is stated that "An instrument that is considered to be sufficiently stronger from two objects acquires comprehensive force when it comes into contact with each other, which leads to the softer one being marked.".

Biasotti and Murdock [13] state that "When two objects begin to interact, the extremely hard object will stamp the surface of the softer object. The relative hardness of the two artifacts, the pressures and motions, and the appearance of the microscopic discrepancies on the object are all factors influencing the character of the generated toolmarks." It is necessary to establish the correlation between a tool mark and the tool that produced it in criminal investigations such as burglaries. For instance, if a burglar chooses wooden or metal bars to force entrance into a home, the marks left by the tool on the doorway are strong evidence of the involvement of that tool for that legitimate purpose at the scene of the crime. If the tool is linked with, or close to, a suspect, it enables for the identification of a link between the accused person and the incidence of the crime.

4. Types of tool marks

Generally, there are three categories of tool marks left by tools on the surfaces they hit. These impressions are produced by the possibility of a compression action, sliding action or cutting action occurring.

A compression impression: Probably the most common and most negative representation of the surface of the tool, caused by pressure, blow or gouge of the tool on the surface of a wood, metal or other surface. Compression is imprinted on softer material when tool surface presses against its surface [14]. For instance,

a screwdriver is most often used to tighten or loosen screws. However, if it is used to pry open a widow, it will leave impressions in the windowsill.

Friction marks (sliding action): The second type is a mark of abrasive wear or resistance left by the tool's sliding or chopping action that creates striations on a marked surface. Friction marks are fine parallel striations and are a characteristic feature left by a tool scraped across a smooth surface, such as dressed wood or metal. It is common to focus on such striations when making bullet and tool mark comparisons. Parallel lines have the potential to be matched using microscopic comparison. There are an infinite number of ways to apply a tool to a surface, and the resultant striations are the effects of every variation. For example, when a crowbar is forced into the area between a door and the front part of the door to force the door wide open, pressure is applied to the tool handle. An abrasion or friction mark is created by forced application of the crowbar. The majority of bull cutter marks on rods or wires, screwdriver scratch marks and knife or axe cut marks are examples of friction mark markings.

Cutting edges are not as commonly used in the commission of crimes as prying tools with blunt edges, so finding marks of cutting tools is not frequent. There is a high significance in cut marks being positively identified with the tool producing them. A cutting impression is a combination of these two impression types, as is found in scissors.

From these three tool mark impression types, both the class and individual characteristics of the tool can be identified; for instance, marks left on a doorway from a pry bar can be matched back to that specific pry bar.

During tool mark analysis, the analyst may discern what type of tool made a particular mark, and whether a tool in evidence is the tool that made it. The tool mark can also be compared to another tool mark to ascertain if the marks were made by similar, or the same, tools.

5. Types of tool marks comparison

A well-known and extensively used forensic methodology is the comparison of tool marks, which is typically regarded to provide convincing trial evidence and facilitate the investigation of a crime. However, there is a great deal of ambiguity as to the uniqueness of such marks and, in particular, the probability of more than one tool replicating a mark. According to Houck and Siegel [15, 16], tool mark examiners need to have a conceptual understanding of how to produce and machine a variety of tools. Limitations on comparative forensics have initiated the need for an objective, as each tool has specific surface characteristics for the identification of tool marks to facilitate scientific research. In 2009 National Academies report, researchers recommend reinforcing the scientific justification for the standards and specifications for the tool mark identification in forensic science.

The forensic principle of comparison explains that only the like can be compared with the notion of comparison. It reinforces the need for samples and specimens to be included for comparison with the objects in question. Therefore, the prime purpose of forensic comparison is to establish which characteristics and specifications of the samples in question obtained from the crime scene (including a tool or a population of reference items, screw bag or plastic bag roll) varied or directly correlate with those obtained from the source on the control item. Comparing features, however, is a deceptively simple process, but understanding what the outcome implies is much more difficult if one does not understand exactly what the characteristics and specifications are or how they were acquired.

Another challenging part of a comparison is to examine the manufacturing patterns associated with the "control" object. The manufacturing process leaves distinctive microscopic striations on the tool's operating surfaces as the marks produced depend not only on the type of tool being used but also on how it is used (as a hammer, or lever or force exerted), the contact position (leading angle or trailing angle) and other factors that may help to identify the metal tools [16].

There are three categories of features that an examiner will need to identify:

- Class features: A combination of features that facilitate the positioning of the sample in a class of related material properties. Champod et al. [17] state that the class characteristics of a tool are usually unique and macroscopic; for example, class characteristics of firearms are correlated with the tensile strength of the weapon and projectile or cartridge steel and the rifling in the firearms barrel that is transferred to the bullet.

- Subclass features: Attributes that are not specific to a particular object but provide some discriminatory practices among groups of tools with features of the very same class. They appear during processing but are not necessarily introduced. Over time, the reference of subclass functionality can evolve. Nichols [4] explains what qualifies a characteristic as subclass: "If one were to examine a cast of the bore of a firearm, such characteristics would have to exist for the entire length of the cut surface. If a certain characteristic appeared after the cut surface had already started, then it would be an imperfection caused by the current process. If it disappeared before the end of the cut surface, then it is gone and by definition of its absence cannot be passed onto the next cut surface. Therefore, the only characteristics capable of being defined a subclass would be those that persist for the entire length of the cut surface."

- Individual/unique characteristic: Individual characteristics relate to the specific characteristics of both the questioned samples and the reference samples, which share a similar origin with a high degree of reliability. Examples of evidence possessing individual characteristics are fingerprints, tool marks and markings on bullets.

Therefore, in order to analyze the results, it is imperative to understand the sets of features and details generated during the production process and then use, how they will be portrayed in a mark and how to differentiate between the different types, as this will determine what you can say about the comparison. The quality of the situation mark in a mark comparison is always the main limitation. Information that may have been visible on a tool may not have been replicated in a mark for certain variables, such as the physical parameters of the material. If they are considerably weaker than the tool, the information of interest cannot be replicated completely.

However, occasionally, the difference will be significant and on occasions may even be to the extent that one expert will say the tool was responsible and the other that it was not the tool. Occasionally, while the difference is apparent, it may be to the degree that one analyst states that the tool was accountable and the other that it was not the tool. With all this perspective, the importance of the independent critical results test of a secondary tool mark expert should not be overlooked. However, this is not always necessary, and in order to settle the debate, a third expert may be required to conduct a verification.

AFTE Theory of Identification (1998) classified four categories of tool examination:

- Identification is the inference that the class traits of two samples appear to be the same and that the individual features are reasonably agreed to conclude that the same weapon was shot. If they agree, for instance, two copper jacketed bullets are found.

- Inconclusive agreement of class characteristics is defined as "the outcome of a comparison in which there is some agreement of individual characteristics and all discernible class characteristics, but insufficient for identification, agreement of all discernible class characteristics due to an absence, insufficient, or lack of reproducibility, agreement of all discernible class characteristics and disagreement of individual characteristics but insufficient for an elimination".

- A substantial disparity between distinguishable class characteristics and/or individual characteristics is triggered by elimination, or exclusion from the analysis. For fired bullet comparisons, an exclusion is usually based on observed differences in some of the general rifling properties.

- In the absence of microscopic marks, "Inappropriate for comparative analysis," appears.

6. Forensic examination of tool marks

The purpose of the analysis and comparison of the tool mark is to determine whether a mark or a series of marks in dispute have been made by a specific tool. Careful examination of the questioned tool mark(s) typically offers descriptions of the class characteristics and size of the tool responsible for making the marks in question, if the tool is damaged and how the tool was used to produce the alleged marks [10]. A tool mark analysis primarily initiates with a morphological examination of the tool and its features. For each tool mark, such as branding, cutting, compression, crimping, engraving, firing, etc., Klees [18] therefore suggests a categorization system to enhance the common classification systems found in the literature and to provide a more standard way. Tool mark analysts are objective and conceptual analysts who seek to assess if they are combating a tool mark and a similar tool. They use their results on the basis of their assessment of the evidence. Tool mark examiners collect information about a piece of evidence in order to establish a hypothesis about what occurred, so that it can be linked with certain other observations and results. Tool mark analysts are unbiased and conceptual analysts who aim to determine whether a tool mark and a particular tool are being countered. They employ their conclusions on the basis of their analysis of the proof. In order to create a hypothesis about what happened, tool mark examiners collect information about a piece of evidence so that it can be combined with other information and conclusions.

6.1 Physical matching

Physical fits, also pointed to as "mechanical fits," can be identified in a massive variety of criminal investigations, even as part of a more detailed instance of the tool mark. A physical fit exam is required when it is imperative to ensure that two or more parts of the product that have been partitioned, broken, cut and often forcibly removed were actually attached or fitted together [19]. Further, Jayaprakash [20] emphasizes on the unique characteristics that make a fundamental paradigm relevant for individualization. Restricted physical comparison literature reviewed

that in the course of the trial, the objective scientific reliability and admissibility of such physical comparisons tend to be regarded with skepticism.

Features participating in physical matching and comparing rendered definitive judgments in patterned evidence that eliminate ambiguity during investigation and also an array of measurable units that comprise the entire pattern area, raising the probability of a pattern of verisimilitude known to trigger infinity that provides evidential justification for individuality [19].

Physical fit examinations fall into four main categories:

- Broken portions that will potentially be refitted, otherwise referred to as "jigsaw" suits.

- Broken items require a thorough tool mark examination such as microscopic comparison and casting in an effort to validate that the parts match together and therefore to form an inference.

- Broken, torn or split objects where knowledge of the manufacture and appearance of marks left on the surface of the material must be taken into account in order to facilitate a fit.

- Objects that were actually built to fit together were perhaps in touch for a period of time. Typically, these examinations require an analysis of what matter has been passed or is a function of the contact.

6.2 Casting

Collection, processing and examination of impression and tool mark evidence are one of the major components of forensics. The disadvantages associated with the selection and preservation factors are an unacceptable mix, creating a negative impression of resources and environmental factors. These restrictions relate to lack of detail, compromise of class perception and individualization of features used to position a particular piece of evidence at the scene of a crime. It is necessary to initially make the best cast possible with the inherent destructive potential of impression and tool mark casting. Occasionally, the circumstances of a crime scene impact the availability of casting techniques, contributing to the continuity of a cast [21].

A wide range of casting materials are often utilized to manufacture casts of tool marks: negative molding, low-melting metal alloys (e.g., wood metal) and silicone rubber. The material that most closely fits the specifications of an efficient casting material is silicone rubber. A tool mark's microscopic detail is carefully repeated; it is impact resistant when kept at room temperature and is comparatively cheap.

The silicone rubber casting material is supplied as a partly polymerized base with which a catalyst must be mixed in order to allow polymerization. Forensic professionals focus on Microsil Silicone Casting Medium to recreate the subtlest tool markings and impressions. Laboratory studies have shown that they are superior to other established flexible silicone casting techniques by substantially improving the visibility of tool marks, firing pin impressions and latent fingerprint lifts. Microsil increases the likelihood of positive acknowledgment.

6.3 Automated system

An automated tool mark identification system uses an acquisition method for the processing of 3D data from tool marks left by tools on the sample surface, a signature generation module for the generation of tool mark signatures from the data

collected and an analysis unit for the comparison of pairs of tool mark signatures in order to obtain a numerical similarity value representing their identical characteristics. The process is carried out with the aid of an integrated computer [22].

6.4 Databases of tool marks

A wide variety of different tool marks are found at the crime scene due to the different shapes and surface where the tool mark is rendered. Bolt cutters, wire cutters or crowbars have been used to break a door in many cases of burglary. These tools can produce marks that appear in various patterns: impressions and striation marks. Therefore, the Netherlands in collaboration with the Dutch Police developed a database for tool marks, known as Tool Mark Imaging System Database (TRAX). The device is designed for collection, restoration and comparing of tool images and their textual descriptors' width, kind of tool mark, etc.) [23].

6.5 Known tool marks test impressions

In practice, the investigator of the tool marks produces negative test tool marks using the suspect tool to compare microscopic surface characteristics between known test tool marks and evidence tool marks. It is recognized that the contrast between a suspected tool and a known test marks is always quicker and more effective than casting or even photography techniques [24]. It is also suggested to use known test tool marks developed in the very same way as the actual tool marks questioned. Traditionally, test tool marks are generated on sheets of soft metal or metal alloy, bars or tubes such as lead, wood alloy and, more recently, lead tape. Firstly, without losing the working surface of the tool, these surfaces are flexible enough to allow test casts with the finest tools. Second, their malleable nature enables the reproduction of the fine scrapes and ridges present on the instrument's working surface in the case of striation marks. Finally, the resulting known test tool marks are accurate, highly detailed, negative impressions of the working surface of the tool [10].

7. Interpretation

Impressions retrieved from crime scene are compared with reference tools to identify the impressions and to determine if they share a common origin. If there is a good fit between the two impressions, it is necessary to categorize the attributes and explain the probability of it being made randomly or on purpose. In the instance of a negative match between features, a careful investigation is required to determine whether the differences are significant or not and if there is a sensible and fair interpretation that can be made.

The forensic examiners can build a complete probative importance of the decision based on such similar and non-similar findings in order to present it as substantial court evidence. This also demonstrates the examiner's extensive knowledge in explaining and analyzing the fabrication process as well as the tool's wear and tear over time.

The following concerns will arise while an expert is doing a mark comparison.

1. Mark the Class and individual characteristic such as substrate and pressure used to create the impression and so forth.

2. Determine the number and characteristics of the impression present on the questioned tool as well as whether or not you would anticipate to see them reproduced in an impression and how well they relate (or do not) to tool attributes.

3. This would be a problem if characteristics like pressed lines, milling, and broaching were designed in such a way that they could appear on numerous tools made in a similar way and be indistinguishable from other tools. Similarly, if qualities like grending or damage breakdown were produced at random and regarded unique, no other instrument would have them.

4. Extraneous particles detected on the surface of impressions retrieved from crime scenes and at the surface of reference tools may be affected by external factors such as the nature of the substrate, the direction and the amount of pressure applied.

8. Evaluation

Evaluation is the framework of a conclusive judgment based on analysis and interpretation in significant detail by weighing what the findings mean in reference to the prosecution and the defense statements. There are (at least) three perspectives about how investigators can report their conclusions.

1. In one approach, the examiner must make claims that represent the balance of probabilities. The investigator either makes a conclusion about the forensic evidence's reliability based on the balance of likelihoods or makes a judgment about the relative probability of the observed findings under alternative theories.

2. The second method necessitates a two-step study.

   - The examiner starts by comparing the objects (tools) to see if there are any significant differences that rule out the possibility of a common source. When identifying characteristics are noted, the investigator decides that the items do not share a common source, a process known as "exclusion."

   - When the objects cannot be differentiated (i.e., the likelihood of a common source cannot be ruled out), the examiner then evaluates the rarity or uniqueness of the shared features as a second step. If the examiner believes that the shared features are so unique that they are peculiar (one-of-a-kind), the examiner may infer (and report) that the items are all from the same source—this conclusion is often called individualization or identification. If the examiner believes the shared characteristics are not identical, he or she could state the uniqueness of the related features or the probability that a random tool of the same kind will have them. Similarly, the examiner may claim unequivocally that the artifacts are indistinguishable or that they "play," without mentioning the match's rarity. Eventually, the analyst may conclude the comparison inconclusive.

3. In a third approach, the examiner will use numbers (e.g., "there is a 99% chance this tool mark was produced by the suspected tool") or words (e.g., "it is extremely likely that these marks were made by the same tool") to draw conclusions about the likelihood that the objects have a similar source. These conclusions are sometimes called source probabilities. This third approach is distinguished from both the first (balance of likelihoods) and the second (two-step analysis) approaches in that it allows the examiner to take a position or make judgments about the prior odds that the items being compared have a common source. To put it another way, the examiner's decision must be based on more than an assessment of the physical characteristics of the tools being comparison.

Additionally, after these two requirements are accomplished, evaluative reports that can be used in court should be generated [25, 26]:

- A mandating authority or party has asked the forensic practitioner to analyze and/or compare material (typically recovered trace material with reference material from known potential sources).

- The forensic practitioner attempts to evaluate findings in relation to specific conflicting propositions established by the unique case circumstances or as specified by the mandating authority.

In court, the results of forensic examinations should be evaluated using a probability ratio relying on the findings, associated data and expert knowledge, case-specific propositions and conditioning information. Since the value of the results is dependent on the case information and propositions, this should be emphasized in the report.

The forensic expert opinion should be carried out on the basis of four precepts first stated in an AFSP paper [27]:

- Balance: in order to reinforce the truth, the expert should accept at least one pair of the hypothesis proposed by the prosecution and defense, and if it is not possible to find a reasonable alternative for any reason, the expert will be able to examine only one proposition, but will make it clear that the strength of the proof cannot be measured.

- Logic: evaluative reports should address the likelihood of the findings given the propositions and relevant background information, rather than the likelihood of the propositions given the findings and background information. Statements that transpose the conditional should not be included in the report.

- Robustness: the opinion of an expert should be resilient and satisfy the reliability standards set by other experts for cross-examination.

- Transparency: by addressing and evaluating hypotheses, examination results, and theoretical facts, it would be necessary for the expert to demonstrate how he came to his inference.

To be these above things, experts need to make it express exactly what they have done and with what technique, what highlights have been thought of and why, what grants have been made and why and, last and most importantly, by unmistakably spreading out an indictment and a defense viewpoint upon which to consider the outcomes. These perspectives will without a doubt be restricting and, in instrument mark assessments, as a rule address the expected wellspring of the mark(s). The indictment view that "the submitted tool made the scene mark" is not hard to define [28].

9. Conclusions

A significant aspect of many forensic investigations is the interpretation of tool marks that may have an impact on a number of disciplines, including anthropology, archaeology and pathology. The reason for the determination of the tool mark is not specific but usually refers to the recognition, adjustment and comparison

of the marks/indentations left on the surface after contact with the tool. Mark evidence involves the analysis of any object where a mark or impression has been rendered during criminal conduct to link the mark with the object or tool that made it.

The AFTE argues that the idea of identification appears contextual, an evaluation that helps researchers to establish protocols that are more precise and detailed. However, new technologies and tools provide the forensic community with a new basis and support to understand, refine and spread the methodology to the experts, which helps to interpret the marks of the tool. Technologies used in surface characterization is constantly changing, and computers are becoming more and more efficient, making it less burdensome for extensive computations, so new methodologies can be more sophisticated. These modern methodologies generally involve first converting a tool mark scan to a digitized striae depth representation in given distances along the mark, collected using a profilometer or similar tool, rather than manually aligning two photographs or imprints of the tool marks. Forensic databases can provide a measure of the accuracy of the identification of certain recognition characteristics, helping to become beneficial in the analysis of evidence. The results of the use of databases will apply not only to court documents but also to organizational activities.

When evidence marks are forwarded for analysis, the investigator shall be given four plausible explanations when assessing the marks: recognition, inconclusive, elimination or unacceptable. Examiners often come down on the side of uncertainty and only accept identification when this conclusion is unanimously accepted. The anticipated qualities of a forensic evaluation are defined by four principles: rational, unambiguous, balanced and rigorous, facilitating the field to transition from a collection of concealed secrets within professionals to a formal body of information from which one can be qualified to be an examiner. Therefore, it is evident that tool mark evaluation and interpretation are complex operations requiring consideration of several intrinsic and extrinsic variables, and so it is not surprising that this is a field of research that has attracted significant interest and discussion over a fairly long history.

Conflict of interest

"The authors declare no conflict of interest."

Acronyms and abbreviations

AFTE	Association of Weapon and Tool Mark Examiners
TRAX	Tool Mark Imaging System Database

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Section 3

Identification Evidence

Chapter 7

Forensic Osteology and Identification

Anil Garg and Nisha Goyal

Abstract

Every human corpse is unique. There are different religions in different parts of the world which adopt a variety of ways to dispose of corpses. Dead bodies can be found unattended, dug up, mutilated by the perpetrators of crimes, and eaten by wild animals in lonely unattended places. In these situations, forensic anthropologists or anatomists are consulted by the state authorities to help them to provide justice to the deceased person. The first and foremost scientific information desired by authorities is identification of the corpse, cause of death of the human body and weapon used, if applicable. Identification can be done by studying the bones of the human corpse during autopsy examination and if unknown skeletal remains are all that is available, examination of each bone is required. Forensic anthropologists or pathologists are asked to identify race, sex and age as important parameters of the identification. In this chapter, we will enumerate various parameters for identification. We will discuss race, age and sex from various bones as part of forensic oesteology.

Keywords: bones, index, skull, femur, ossification centre, race, age, sex, skull, pelvis, mandible, rhomboid fossa

1. Introduction

The human corpse is more than a utilitarian object; it has sacred meaning. Every religious faith has beliefs pertaining to the treatment of corpses and there are laws that govern the treatment and the burial of the dead. While these laws have recognized the corpse's instrumental value as an object for scientific study, clinical teaching and commercial gain, they generally accommodate the desire to respect the remains [1].

Forensic experts, in particular anthropologists, frequently are asked to examine unknown corpses before final rituals for identification in medico-legal cases. Identification is the determination of the individuality of a person. This can be for either a living or dead person. Various parameters for identification of human dead bodies are enumerated below.

1. Race

2. Sex

3. Age

4. Stature
5. Teeth
6. Hair
7. Religion
8. Fingerprints
9. Footprints
10. Tattoos
11. Scar marks
12. Anthropological factors

A thread that binds parameters such as race, sex, age and stature is human osteology or forensic Osteology. Bones and teeth of the skeleton resist putrefaction or decay. Hence they are a cornerstone for the determination of individual existence. Scientists employ their knowledge of the human skeleton in interpreting the bones and thus help in identification.

Human forensic osteology is the study or application or knowledge of human bones in the field of forensic science to assist the administration of justice.

In this chapter, we will mainly consider race, age and sex parameters.

2. Race

Human bone measurements play vital role in the determination of race. The important bones that are useful for race determination are the skull and the long bones of the limbs. Various indexes are given for these.

Index is defined as a percentage expression of the ratio of a smaller dimension over the larger one.

2.1 Cepahalic index

The cephalic index (CI) is calculated from the skull according to the following equation:

$$(\text{cephalic width}/\text{cephalic length}) \times 100 \tag{1}$$

Cephalic length is the distance between the most anterior and posterior point of the outer table of the skull or occipitofrontal diameter (OFD). Cephalic width is the distance between the outer skull tables at the widest points of the skull or biparietal diameter BPD [2]. Cohens [3] classifies race on the basis of cephalic index as dolichocephaly (long headed) up to 75.9 e.g. Pure Aryans, Caucoids and Negroids, mesocephaly (round headed): 76.8–80.9 e.g. in few Caucoids (Europeans) and Mangoloids, and brachycephaly (Short headed): 81.0–85.4 e.g. Mongoloids, with hyperbrachycephaly exceeding 85.5 e.g. Kyushu of Japan.

2.2 Nasal index

Nasal anthropometry is the study of proportion, shape and size of the nose in human beings. The nasal index is the ratio of nasal width to nasal height multiplied by 100.

$$\text{Nasal Index} = \text{Nasal Width} * 100/\text{Nasal Height} \quad (2)$$

It also exhibits sexual differences and has become an important tool in forensic studies. The general shape of the nasal base has long been broadly classified as the leptorrhine or long/narrow nose, the mesorrhine or medium nose and the platyrrhine or short broad nose [4].

Leptorrhine: Lesser than 70. Caucoids
Mesorrhine: 70–85 Mangloids
Platyrrhine: greater than 85; Negroids

In a study in Nigeria on Igbo and Yoruba males and females, it was observed that both had the same type of nose Platyrrhine, but differences still existed. The report showed that the Igbo males and females had mean nasal indices of 95.8 ± 0.44 and 90.8±0.61 respectively while the Yoruba males and females had mean nasal indices of 90.0±0.38 and 88.1±0.47 respectively. The Igbo (Total) had mean nasal indices of 94.1 ± 0.37 while the Yoruba (Total) had mean nasal indices of 89.2±0.30. The mean nasal indices of Igbo males and females were significantly higher than those of Yoruba males and females [5].

3. Age

Age determination from humans is one of the important tasks desired by law enforcement agencies for medico-legal cases. Absolute or chronological age is the number of years an individual has lived since birth. In other words, it is the age that is mentioned on the passports or other important documents of the person. Biological age is the age of the person gauged from the physical wellbeing of the person [6]. Environment, health conditions, exercise, yoga and healthy eating habits affect the biological age, not the chronological age. The difference between chronological and biological age is minimal in juveniles, but it increases afterwards [7].

In fetuses and children, age can be estimated from the appearance of ossification centres, development of bones and eruption and calcification of the teeth. There are approximately 806 ossification centers at the 11th prenatal week, 406 ossification centres at birth and 206 bones in the adult. The ossification centres enlarge in size and joints to nearby ossification centres and thus give rise to the bones in the adult skeleton [7]. A fetus' age is best given in lunar months although it is also given in weeks of pregnancy. In decomposed fetal bodies, it is best to have the fetal body X-rayed [8]. But in skeletonised fetuses, various bones dissociate, thus X-rays are not helpful. The presence of the primary ossification centre of the talus, calcaneum, cuboid and the secondary ossification centre in the femur and tibia around the knee joint point toward full term pregnancy [9]. The major ossification centres appear [10] as follows:

At Birth: calcaneum, talus, femur distal end, tibia proximal end, cuboid, humerus head.
At Second Month: capitate, hamate, lateral cuneiform.
At 3 month: femus head, capitulum, tibia distal end.
At 6th month: fibula distal end.
At 7th month: humerus, greater tuberosity, radius distal end.

At 10th month: triquetrum.
At 11th month: third finger-first phalanx, first toe-second phalanx.
At 12th month: second finger-first phalanx, fourth finger-first phalanx, first finger-second phalanx.
At 13th month: third toe-first phalanx, second metacarpal, medial cuneiform.
At 14th month: fourth toe – first phalanx, second toe – first phalanx fifth toe-second phalanx.
At 15th month: third metacarpal, second toe-second phalanx, fifth finger-first phalanx.
At 16 month: fourth toe-second phalanx, fourth metacarpal.
At 18th month: fifth metacarpal, second, third and fourth finger-second phalanx.
At 20th month: first toe-first phalanx, middle cuneiform [10].

Fetal age can be determined by crown heel length (CHL). According to Hasse's rule which is a crude method to determine fetal age, in the first 5 months of fetal life, the square root of crown heel length measured in cm, will give the age of fetus in months. As with the Morrison rule, after five months of fetal life, the crown heel length in cm is divided by the number five to reach the fetal age in months.

In the mandible and maxilla, the primary centre of ossification appears at 6 weeks, while in frontal bones ossification begins in 6–7 weeks, and in the temporal bone, ossification appears in 7–8 weeks. In occipital bone, ossification centre appears in 8–10 weeks of intrauterine life [11].

The appearance of secondary ossification centre [11] appear as shown in **Table 1**.

In adult skeletonised remains, epiphyseal closure or fusion is more commonly seen than ossification centres. This process of closure usually starts from 12 to 14 years and chronologically happens earlier in females as compared to males.

Stevenson [12] described four stages of fusion as follows:

1. First Stage or No fusion: On gross examination of skeletal remains, there is a clear cut hiatus in between the epiphysis and diaphysis. The margins of the epiphysis and diaphysis is serrated or saw-toothed.

2. Second Stage or Beginning of fusion: There is a clear cut line in between the epiphysis and diaphysis. The first phase hiatus is replaced by formation of new bone leaving only a line of separation. The saw-toothed appearance of margins in the epiphysis or diaphysis as evident in the first stage, is also blurred or lost.

3. Third stage or recent union: The clear cut line in the second stage is as appreciable as the fine line. This stage is sometimes difficult to appreciate.

4. Fourth stage or stage of complete union: This stage represents complete fusion. Sometimes, a very faint epiphyseal line is appreciable throughout life.

Loth [13] described that the order of epiphyseal closure of various joints is as follows. First the elbow is followed by the hip, followed by the ankle, followed by the knee, followed by the wrist, and last in the shoulder joint.

3.1 Sternum

The sternum is made of the manubrium, body of the sternum and the xiphi-sternum. The body of the sternum is the middle-most part and is composed of four parts. The fusion of the sternum is variable. Different authors have expressed

Sr. No.	Bones Parts	Age
	Shaft	Birth
	Medial Epicondyle	12–14
	Lateral Epicondyle	19–20
	Humeral shaft	Birth
	Humeral head	2–6 months
	Humeral Capitulum	By 1st Year
	Humeral Greater Tubercle	6 months-2 years
	Humeral Lesser Tubercle	4+ years
	Humeral medial epicondyle	4+ years
	Humeral Trochlea	8 year
	Humeral Lateral Epicondyle	10th year
	Radius Shaft	Birth
	Radial distal Epiphysis	1–2 years
	Radial head	5th year
	Radial styloid process	8th year
	Ulnar shaft	Birth
	Ulnar distal Epiphysis	5–7 years
	Ulnar styloid process and olecranon	8–10 years
	Pelvis	Birth
	Femoral shaft	Birth
	Femoral distal epiphysis	Birth
	Femoral Greater trochanter	2–5 years
	Femoral lesser trochanter	7–12 years
	Tibial Shaft	Birth
	Tibial proximal epiphysis	Birth
	Tibial Medial Malleolus	3–5 years
	Tibial Tuberosity	8–13 years
	Fibular Shaft	Birth
	Fibular distal epiphysis	9–12 years

Table 1.
Showing appearance secondary ossification Centre from bones.

different views. Sternebra are numbered from upwards to downwards as 1 to 4. Sternebra 3 fuses with 4 between the ages 4 and 15. Sternebra 2 fuses with 1 and 3 by the ages of between 11 and 20. The manubrium fuses with sternebra 1 by between the ages of 15 and 25 years [7]. The xiphoid fuses with sternebra 4 in older age.

Garg [14] conducted a radiological study on 150 living subjects by doing lateral view X-rays of the sternum in the age group of 35–65 years whose exact age is known by available official documents and where the entire sternum was intact without disease and deformity. He concluded that complete fusion of the xiphisternun with the body of the sternum occurred by 56–59 years and only 40% manubrium fused with body of sternum by 65 years.

Mean Closure Value	Mean Age	SD	Range	Age Category
0.4–1.5	28.6	13.08	15–40	Juvenile-young adult
1.6–2.5	43.7	14.46	30–60	Young-middle adult
2.6–2.9	49.1	16.40	35–65	Young-middle adult
3.0–3.9	60	13.23	45–75	Middle-old adult
4.0	65.4	14.05	50–80	Middle-old adult

Table 2.
Showing estimation of age by cranial suturel closure [6] by mean Acsadi score.

3.2 Cranial sutures

Cranial sutures are extensively studied by different authors for age estimation. Cranial Sutures usually fuse in adult life except the metopic suture. The metopic suture fuses by the age of 1 to 4 years. The fusion of the cranial suture in adult life is studied both endocranially and ectocranially. Cranial sutures are assessed in three sections or parts: palate is also studied along with endocranial and ectocranial study of cranial sutures.

Recently also the method devised by Acsadi and Nemeskeri [6] has been widely used. They studied sagittal, coronal and lambdoid sutures for the purpose of age estimation. They divided the coronal suture into three parts, the sagittal suture into four parts and the lambdoid suture into three parts – in total 16 sections. Then they studied closure of sutures and gave scores as follows:

Score 0: Open suture.
Score 1: Suture line is closed but clearly visible and continuous.
Score 2: Suture line is thinner and may be interrupted by complete closure at places.
Score 3: At the suture line, only pits are available.
Score 4: Suture is completely obliterated.

Each of 16 sections described above was examined and awarded scores and a mean value was calculated, then that mean closure value was compared by the **Table 2** given below and the mean age was calculated and the age category was noted.

In young adult life, the incisival palatine suture is closed with activity seen at transverse and posterior palatine suture. The anterior palatine remains completely open. In middle-aged adult life, the incisival transverse and posterior palatine suture are closed. The interior palatine remains partially open. In old age, all palatine sutures are fused [15].

There are many more bones from which age can be found. The bones described here are the bones which are frequently examined by forensic anthropologists.

4. Sex

In humans, it is very difficult to determine sex from skeletal remains. Until adolescence, the human skeleton is immature and starts maturing at puberty or adolescence and thus attains complete maturity in adulthood. Thus, sex determination with accuracy in young to adult life is difficult as many factors overlap.

Sr. No	Bones Available	Accuracy of Sex determination by Krogman [16]	Accuracy of Sex determination by Stewart [17]
1	Entire Skeleton	100%	90–95%
2	Pelvis + Skull	98%	—
3	Pelvis + Long Bones	98%	—
4	Skull Alone	98%	80%
5	Pelvis alone	95%	—
6	Long bones only	80%	—
7	Skull + mandible	—	90%

Table 3.
Showing accuracy of sexual identification from bones.

Krogman [16] studied a sample of 750 adult skeletons (white and black, male and female) from the Harmann-Todd collection and Stewart [17] also determined sex and found as shown in **Table 3**.

Sex can be determined by two methods – morphological and metric. The morphological method of assessing sex is by reference to the differences in skeletal remains on the basis of gross examination. It relies on the specific bony traits and muscular markings etc. to differentiate the skeletal remains. The advantage of the morphological method is that sex-specific bony characteristics remain unique in spite of population variations. But gross examination of morphological characteristics of the skeleton has disadvantages such as inter- and intra-observer errors, observer experience, and standardization and statistical analysis problems. This gross morphological method of determining sex is challenged by modern morphological methods such as the geometric morphometric technique [18] and elliptical Fourier analysis [19].

Earlier in the gross morphology technique, the skeletal remains are observed in two dimensions and now by reference to the geometric morphometric technique, the shape differences are first observed and then quantified in three dimensions digitally. Thus, this technique reduces the inter- and intra-observer errors. This new technique works well at a population level but it is very difficult to apply to individuals. Nowadays, a number of sex dimorphic characters are studied morphometrically and then statistically analyzed by discriminant function analysis, logistic regression and neural networking.

4.1 Pelvis

4.1.1 Morphological assessment

The human pelvis consists of 3 bones namely the hip bone, the sacrum and the coccyx. The hip bone consists of 3 parts i.e. the ilium, the ischium and the pubis. The pelvis is the most sexually dimorphic bone of the human skeleton as it determines the sex very accurately. The pelvis is the most widely studied bone to determine sex from unknown skeletal remains. As Krogman [20] has identified, the pelvis can identify correct sex in 95% (**Table 3**) of cases from unknown skeletal remains. **Table 4** enumerates classical morphological sex differences from pelvis.

Phenice [21] studied 275 adult individual already sexed pelvises from the Terry collection with three visual traits named the ventral arc, the subpubic concavity and the medial aspect of ischiopubic ramus and found sex with 95% accuracy. He also

Sr. No	Characters of bone	Male	Females
1	Pelvis as a whole	Massive, rugged, marked muscle sites	Less massive, gracile, smoother
2	Symphysis	Higher	Lower
3	Subpubic angle	V-shaped (<90°)	U-shaped: rounded;broader divergent obtuse angle (>90°)
4	Subpubic shape	Convex	Concave
5	Pubic bone shape	Triangular	Rectangular
6	Ventral arc	Absent, not well	Well defined
7	Obturator foramen	Large, often ovoid	Small, triangular
8	Acetabulum	Large, tends to be directed laterally	Small, tends to be directed anterolaterally
9	Greater sciatic notch	Smaller, close, deep	Larger, wider, shallower
10	Ischiopubic rami	Slightly everted	Strongly everted
11	Sacroiliac joint	Large	Small, oblique
12	Auricular surface	Raised	Flat
13	Postauricular space	Narrow	Wide
14	Preauricular sulcus	Not frequent	More frequent, better developed
15	Postauricular sulcus	Not frequent	More frequent, sharper auricular surface edge
16	Ilium	High, tends to be Vertical	Lower, laterally divergent
17	Iliac tuberosity	Large, not pointed	Small or absent, pointed or varied
18	Sacrum	Longer, narrower, with more evenly distributed curvature; often 5 or more segments	Shorter, broader, with tendency of marked curvature at S1–2 and S2–5; 5 segments the rule
19	Pelvic brim,or inlet	Heart shaped	Circular, elliptical
20	True pelvis, or cavity	Relatively smaller	Oblique, shallow, spacious

Table 4.
Shows classical morphological sex differences from pelvis.

found that the ventral arc is the least ambiguous and medial aspect of the ischiopubic ramus as the most ambiguous trait among the three traits studied.

Kelley [22] observed after applying the Phenice technique in 392 mature pelvis of both sexes from collection from University of California, Berkeley and Sacramento State University that the Phenice method of sexing with three virtual traits is very reliable and also found that fewer intermediate features are present with the ventral arc and if intermediate features are present in two or all the three traits, then the pelvis is of the female sex.

Bruzek [23] found 95% accuracy in sex determination by using a new visual method taking into account five traits of the hip bone, namely the preauricular sulcus, the greater sciatic notch, the composite arch, the morphology of the inferior pelvis and ischiopubic proportions.

Bytheway [24] studied thirty-six traits digitally of 200 African and European American male and female adult humans' coxae and showed that sex and size have a significant effect on shape for both European Americans. The discriminant analysis

shows that sexing accuracy for European Americans is 98% for both males and females, 98% for African American females, and 100% for African American males.

Iscan and Derrick [25] developed a gross assessment method for sex determination using the sacroiliac joint with three structures which included the post-auricular sulcus, the postauricular space and the iliac tuberosity. They found these to be highly accurate in determining sex.

4.1.2 Metric assessment

There are multiple studies suggesting various indices to access sexual dimorphism.

4.1.2.1 Turner pelvic index

Turner [26] described the shape of the pelvic inlet based on the conjugate diameter (anteroposterior diameter) and transverse diameter of pelvic inlet. It is also known as the Brim Index.

$$\begin{aligned}\text{Brim Index} &= \text{Turner Pelvic Index} \\ &= (\text{Conjugate diameter}\,(\text{anteroposterior diameter} \\ &* 100/\text{transverse diameter of pelvic inlet})\end{aligned} \tag{3}$$

On the basis of the index, Turner divided inlet into three classes as follows
Platypellic = less than 90 (90 not included)
Mesatipellic = 90 to 95 (both 90 and 95 included)
Dolichopellic = greater than 95 (95 not included)

He found that the brim index in males is somewhat lower than in females.

4.1.2.2 Ischiopubic index (Washburn index)

The ishchiopubic index is given by Washburn [27]. It is calculated as follows

$$(\text{Pubic length} * 100/\text{Ischial Length}) \tag{4}$$

Both lengths can be measured with a vernier caliper from the point in the acetabulum where the ilium, ischium and pubis fuse, which may be a notch, raised or irregular area in the acetabulum. The caliper should be held parallel to the long axis of the bone. The author also suggested that the index alone will determine sex from skeletal remains of any one particular population race by up to over 90%. However, overlapping may occur in the skeletal remains of different races as found in white males and black females (**Table 5**).

Population	Male	Female
White	73–94 (83.6 ± 4)	91–115 (99.5 ± 5.1)
Black	71–88 (79.9 ± 4)	84–104 (95 ± 4.6)

Table 5.
Showing ischiopubic index in white and blacks.

4.1.2.3 Sciatic notch index

The sciatic notch index is given by dividing the hundred times width of sciatic notch with its depth.

$$(\text{Width of the sacrum}/\text{diameter of sacrum}) * 100 \quad (5)$$

In adult males: 145; in adult females: 166.
In the male fetus: 4–5; in the female fetus: 5–6.

4.1.2.4 Chilotic line index

The chilotic line index is obtained by dividing the hundred times length of the sacral part of the pectineal line with the pubic part of pectineal line.

$$(\text{Sacral part of pectineal line}/\text{pubic part of pectineal line}) * 100 \quad (6)$$

In males: the CLI is greater than 100, In females: the CLI is less than 100.

These indexes are not used routinely. Nowadays, discriminant function analysis is used by anthropologists. This was first used by Howells [28]. He worked on Gaillard's skeletal collection (75 males, 69 females) and took four parameters, ischial and pubic lengths and the index obtained from it, he took four measurements of the greater sciatic notch and acetabular region. These included sciatic height, cotylosciatic length (shortest distance from acetabular rim to greater sciatic notch), cotylopubic length (from acetabular rim to pubic symphysis) and the difference between SS-SA, in which SS is the distance between the anterior superior iliac spine and the closest point on the greater sciatic notch, and SA is the distance between the anterior superior iliac spine and the closest point on the auricular surface (**Table 6**).

In another study, Dixit [29] observed twelve measurements and five indices from 100 human hip bones of unknown sex of Indian origin. Each of the hip bones was classified as male, female and intermediate on the basis of morphological characters. Afterwards discriminant function analysis was done and it was observed that sex can be accessed with greater accuracy from parameters such as the

From Howells [28]	**Male**		**Female**	
Dimension (mm)	**Mean**	**S.D.**	**Mean**	**S.D.**
X1 Ischial length	96.9	5.65	89.3	5.00
X2 Pubic length	93.2	6.48	97.0	5.31
X3 Ischiopubic index	96.2	3.81	108.7	4.18
X4 Sciatic height	41.0	4.80	47.1	5.32
X4 Cotylosciatic length	40.1	3.13	37.2	3.97
X5 Cotylopubic length	29.7	2.71	24.8	2.63
X6 SS-SA	1.4	3.88	−7.7	4.33
Discriminant Function Formulae			Section Point	% Correct
$Y = 0.7717X1–0.636X2$			11.3	97.8
$Y = 0.8285X6 + 0.517X7–0.1148X4–0.1819X5$			9.2	93.1
$Y = 0.4514X6 + 0.3253X7 + 0.6071X1–0.0993X4–0.1345X5–0.05421X2$			9.3	96.5

Table 6.
Showing discriminant function coefficients for determining sex from the Os Coxa.

acetabular height (vertical diameter) and indices 1 (total pelvic height/acetabular height), 2 (midpubic width/acetabular height) and 3 (pubic length/acetabular height). Pelvic brim depth, minimum width of ischiopubic ramus and indices 4 (pelvic brim chord * pelvic brim depth) and 5 (pubic length * 100/ischial length) were also good discriminators of sex. The remaining parameters used in the study were not significant as they showed a lot of overlap between the male and female categories. The results indicated that one exclusive criterion for sexing was index 3 (pubic length/acetabular height).

4.2 Sacrum

The sacrum is a large flattened triangular bone formed by the fusion of five sacral vertebrae and forming the posterosuperior part of bony pelvis. It articulates on either side with the corresponding innominate or hip bone forming sacroiliac joint. Morphological and metric differences of sex determination are given in the **Table** 7.

4.2.1 Sacral index

The sacral index [30] is given by dividing the hundred times length of anterior superior breadth of the sacrum at the first sacral vertebrae with anterior length of sacrum. The anterior length was measured along the midline from the antero-superior margin of the promontory to the middle of antero-inferior margin of the last sacral vertebra. The anterior superior breadth was measured between the lateralmost points of the ala of the sacrum.

$$\text{Sacral Index} = (100 * \text{Anterior superior breadth of sacrum}/\text{Anterior length of the sacrum}) \tag{7}$$

The study [30] also calculated the demarcating point (DP) which increases the accuracy by 100%. The range of sacral index in male is 80.7–106.4 and in females is 93.1–108.8 and DP for sacral index in males is less than 90.29 and in females is greater than 112.43.

In a study [31] done on 150 fully ossified dry human sacrums (59 male and 91 females), it was observed that the mean straight length of sacrum in the male and in the female was 104.27±5.76 mm and 92.82±7.59 mm respectively. The mean width of sacrum in the male and the female was 99.51±5.80 mm and 102.98±6.69 mm respectively. The mean sacral indices were 95.42±3.14 and 111.27±7.66 in males and females respectively.

Sr. No	Trait	Male	Females
1	Size and shape	Longer, Narrower	Shorter, wider
2	Curvature	More evenly distributed	Curvature not seen in the upper half, lower half curves suddenly
3	Sacral Promontry	Well marked	Less marked
4	Body of first sacral vertbra	Larger	Smaller
5	Sacroiliac articulation	Large, Extends to 2.5to 3 vertebrae	Small, Extends to 2 to 2.5 vertebrae

Table 7.
Showing difference in human sacrum with respect to sex.

4.2.2 Kimura base wing index

Kimura [32] examined 300 sacrums (103 Japanese sacra from the Yokohama City Medical School, 100 American whites and 97 American blacks) and obtained the transverse width of the sacral base, transverse width of the wing and the index as follows.

$$\text{Kimura base wing index} = (100 * \text{transverse width of wing/Breadth or transverse width of Ist sacral vertebra}) \quad (8)$$

The Kimura base wing index is also known as the Alar Index. In males it is less than 65 and in females: it is more than 80.

Patel [33] observed that the sacral index results are more reliable than the Kimura base wing index.

Valojerdy studied 153 dry human sacrums of Indian origin [34], and found that the size of the articular surface was studied in sacro-iliac joints. He found that the articular surface on sacral and iliac surfaces in males is longer and larger in surface area than in females.

4.2.3 Corporo-basal index

The corporo-basal index is the transverse diameter of body of the sacrum S1 when the breadth of the sacrum is 100

$$\text{Corporo} - \text{basal Index} = (\text{Transverse diameter of body of S1} * 100/\text{Maximum breadth of sacrum}) \quad (9)$$

Maddikunta [35] studied 60 adult sacrums from Telengana, India (27 male, 33 female) and calculated the corpora-basal index and demarking point and observed that the range in males is 39.0–53.77 and in females is 27.43–32.67 and the demarking point in males and females is >57.81 mm and <32.02 mm respectively.

4.3 Skull

Th skull is very important for aging and sex differentiation. Sexing can be done with the help of morphological as well as metric characters. As Krogman [16] has identified, if only the skull is available from bony remains, sex can be given correctly up to 98% of the time (**Table 3**). Differences in male and female skull on the basis of morphological characters are given below in **Table 8**.

Buikstra et al. [15] concluded that five traits of the skull should be regarded as able to differentiate sex:

i. Robusticity of the nuchal crest,

ii. Size of the mastoid process,

iii. Sharpness of the supraorbital margin,

iv. Prominence of the glabella, and

v. Projection of the mental eminence

S. No	Feature	Male skull	Female skull
1	General appearance	Larger, heavier, rugged, marked muscular ridges	Smaller, lighter, walls thinner, smoother
2	Forehead	Receding, irregular, rough, less rounded	Vertical, round, full, infantile, smooth
3	Cranial capacity	More capacious (1450–1550 cc)	Less capacious (1300–1350 cc)
4	Glabella	Prominent	Less prominent
5	Supraorbital/Superciliary ridge	Prominent	Less prominent
6	Frontonasal junction	Distinct angulation	Smoothly curved
7	Orbits	Square, rounded margins, small	Rounded, sharp margins, large
8	Frontal and parietal eminence	Less prominent	Prominent
9	Zygomatic arch	Prominent	Not prominent
10	Occipital area (Muscle markings and protuberance)	Prominent	Not prominent
11	Mastoid process	Large, round, blunt	Small, smooth, pointed
12	Digastric groove	Deep	Shallow
13	Condylar facet	Long, narrow	Short, broad
14	Palate	Large, U-shaped, broad	Small, parabolic
15	Foramen magnum	Relatively large, long	Small, round
16	External auditory meatus	Bony ridge along upper border prominent	Often absent

Table 8.
Showing morphological differences in male and female skulls.

The above features are examined independently and scores 1 to 5 is given. A score of 1 is definitely female, 2 is probably female, 3 is ambiguous, 4 is probably male and 5 is definitely male.

Rogers [36] examined 46 identified skulls from a cemetery in Belleville, Canada. He examined 17 morphological features of the skull commonly used to determine the sex of unknown skeletal remains. He observed that traits such as nasal aperture, zygomatic extension, malar size/rugosity, and supraorbital ridge are the most useful; chin form and nuchal crest are the second most useful followed by mastoid size as a tertiary consideration; nasal size and mandibular symphysis/ramus size rank fourth; forehead shape ranks fifth; and palate size/shape are sixth. Skull size/architecture provides an internal standard to assess the relative sizes of other traits.

4.4 Mandible

The mandible is a very important bone in sex determination. Stewart [17] observed that if the mandible along with the skull are the only available bones out of skeletal remains, sex can be determined with 90% accuracy. The projection of mental eminence is one of five characteristics suggested by Buikstra and Ubelakar [15] for sex discrimination (**Table 9**).

Loth [37] examined a sample of 300 mandibles from the Dart collection with known sex. 100 showed bony pathologies and tooth loss. Thus these pathological samples of mandibles were not considered in main study. Of the remaining 200,

S. No	Feature	Male mandible	Female mandible
1	General appearance	Larger, thicker	Smaller, thinner
2	Chin (symphysis menti)	Square or U-shaped	Rounded
3	Angle of body with ramus	Less obtuse (< 125°), prominent	More obtuse, not prominent
4	Angle of mandible (gonion)	Everted	Inverted
5	Body height at symphysis	Greater	Smaller
6	Ascending ramus	Greater breadth	Smaller breadth
7	Ramus flexure	Rearward angulation of the posterior border of ramus	Straight ramus
8	Muscular markings	Prominent	Not prominent

Table 9.
Showing morphological differences for sex determination from mandible.

normative samples consisted of 116 males and 84 females. After careful macroscopic examination, Loth discovered a new trait known as flexure at the level of the molar occlusal surface in adult males. It is a male developmental character that is developed after adolescence. Females retain the straight juvenile shape of the mandibular ramus. Since male develop distinct angulation of the posterior border of mandibular ramus, it usually appears near the neck of condyle or along with gonial prominence or eversion. In the sample of 200, sex was able to be determined in 99% of mandibles. The same parameter was also applied to discarded or pathological samples of mandibles; it yielded 91% accuracy in sex determination.

Kemkes-Grottenthaler [38] investigated the reliability of two mandibular traits: ramus flexure and gonial eversion. The study was done on two samples, one of forensic (N = 153) and one of archeological provenance (N = 80). It was observed that for ramus flexure, male accuracy was only 66%, while female accuracy was even lower (32%). Overall accuracy was 59%. For gonial eversion, a similar picture emerged (75.4% for males, 45.2% for females and 69.3% overall accuracy). Both these indicators are affected by intra- as well as inter-observer bias.

With the development of multiple discriminant function analysis, formulae for various populations have been published taking into consideration various intercorrelated dimensions as well as the degree of difference between sexes.

4.5. Scapula

The scapula is not widely used for sex discrimination. However, a few studies are available. Iordanidis [39] has taken into account scapular height and breadth, total length of the spine and width of the glenoid cavity, calculated by upper and lower limit for discriminating between each sex (**Table 10**).

Traits	Male	Female
Scapular Height	>157	<144
Scapular Breadth	.106	<93
Total Length of spine	>141	<128
Width of glenoid cavity	>29	<26

Table 10.
Showing sex determination by scapula measurements (from Iordanidis [39]).

4.6. Clavicle

The clavicle is also used very rarely in the discrimination of sex from skeletal remains. However, recently a number of authors have show, interest in the clavicle for sex discrimination.

The costoclavicular (rhomboid) ligament joins the first rib anterior to the clavicle to give stability to the pectoral girdle. During this process, sometimes it leaves a depression known as the rhomboid fossa or tubercle or roughened impression, deep fossa or no trace at all. Rogers [40] found correlation with the rhomboid fossa and sex. If the rhomboid fossa is present on the clavicle, the clavicle is of male sex.

5. Conclusions

Forensic osteology is an important part of identification for the criminal justice system. In the past, we talked about morphological ways of sexing more then metric methods and now neural networking is coming for sexing. Further studies must be done so that we can enrich our knowledge.

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Conflict of interest

No conflict of interest is present.

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Chapter 8

Obstetric Markers as a Diagnostic Forensic Tool

Adithi Shetty and B. Suresh Kumar Shetty

Abstract

The field of Forensic diagnostics is evolving very rapidly keeping in pace with the emerging technology in the various fields. Several biomarkers up to the molecular level have been discovered which aid in solving cases. Pregnancy diagnosis from traces of blood could aid in solving cases of finding a missing pregnant lady or illegal abortions. But the challenge posed could possibly be the minimal amount of blood obtained for diagnosis. Here comes in the role of RT PCR diagnosing mRNA which is pregnancy specific, i.e., for hPL and beta hCG. The additional advantage would be that a small quantity suffices. Even if the blood stain is dried and degraded, the detection rate is good. This could add weightage to the investigation as a vital clue or change the course of investigation**.** The other areas of application of obstetric biomarkers are sexual assault, maternal substance abuse and paternity testing.

Keywords: Crime scene, mRNA, bloodstains, RT-PCR, noninvasive paternity testing, STR, SNP

1. Introduction

Forensic diagnostics is one area which is developing in an extremely fast rate and has changed the way in which investigations are handled. The principles of the various disciplines -immunology, biotechnology, biochemistry, molecular biology etc. is integrated into the various diagnostic modalities developed to solve a case scientifically in order to achieve the final result [1]. But pregnancy diagnostics is one area in forensics which has a lot of gray areas.

The possible cases could be sexual assault of a pregnant woman, criminal miscarriages, feticides, drug and alcohol abuse, paternity detection. Crime scenes often yield samples – biological human body fluids – vaginal fluid, saliva, skin and liquid tissues- semen and blood. These provide the necessary genetic material which could help in firstly, establishing the identity of the concerned individual and also aids in establishing the cellular origin of the concerned material. Hence, the investigators need appropriate and effective tools to aid in detection of cellular origin of the sample [2].

Bloodstains from pregnant women can be diagnosed using obstetric markers and aid in solving cases related to criminal miscarriages, feticides, infanticides and identification of missing pregnant women. The earlier markers worked on the principle of immunodetection of protein specific for pregnancy needing large amount of bloodstains for detection with lower sensitivity rates. The possibility of utilization of placental derived mRNA in plasma of the mother using RT-PCR in detection of blood stains proved to be promising [3].

Alcohol intake during early pregnancy causes a teratogenic effect on the fetus is a known fact. But unfortunately there is no well documented screening method in pregnancy to detect alcohol use. One marker that is sensitive and specific which is gaining importance is Phosphatidylethanol 16:0/18:1 [4]. The other markers being evaluated are non-oxidative direct ethanol metabolites such as ethyl glucuronide (EtG), ethyl sulphate (EtS) and fatty acid ethyl esters (FAEEs) [5].

Likewise, use of obstetric markers in assistance to solve the various forensic cases have been studied for some time now. Earlier the use of these markers were downplayed, but now they are having an emerging role in aiding to solve crimes.

2. Obstetric markers for bloodstains

2.1 History of the various biomarkers

Bloodstains at the site of crime are often obtained. But is there a way to detect if the bloodstains belong to a pregnant woman? The answer is yes. But, of course a lot of research has been done in order to find the "ideal" detection method. The base for the research is the use of methods to detect hormones or associated proteins specific for pregnancy and puerperium.

2.1.1 Pregnancy Hormones

The earliest attempts for detection of **pregnancy hormones** in bloodstains started in the 1900s. The first hormone researched was choriogonadotropins hormones as it was one of the ways to establish that the bloodstain belonged to a pregnant lady. In 1932, Goroncy invented a test based on the Aschheim-Zondek test, which detected pregnancy. He made modifications to it and thought that it could prove valuable as an investigation. Although the drawback of this test was frequent false negative results. So it was not a very effective method to detect if bloodstains belonged to a pregnant woman.

After that many techniques were employed for the same- Hemagglutination Inhibition test, crossed electroimmunodiffusion procedure. In the Hemagglutination test, the test cells are hCG sensitized RBCs. Suspected stain extract with the control was incubated with antihCG titres and then addition of test cells was done. But this method was cumbersome and was discarded.

2.1.2 Pregnancy Specific Proteins

The detection of **pregnancy specific proteins** in bloodstains to determine the pregnancy status was also attempted. The 4 proteins detected were – PAPP A, HPL, SP1 and Pregnancy associated alpha 2 glycoprotein. In 1971, Bohn reported that by Immunodiffusion, four proteins could be detected in pregnant serum. The first component was identical to hPL, one another was an a2-glycoprotein, while the remaining two were glycoproteins. In the pregnancy serum, four antigens were detected by Gall and Halbert in 1972. They named them as pregnancy associated plasma proteins A, B, C and D, or PAPP-A, -B,-C and –D. In 1973, Bohn coined the term SP1 for the pregnancy specific 8 – glycoprotein [6]. A lot of research was done to establish which of the hormones or proteins were the best indicator of pregnancy with high specificity [7].

Strejc et al. published a paper in 1989 wherein he detected SP 1 by the process of Immunoprecipitation using self-produced antiserum and it was considered a reliable marker, though, the drawback was that the detection was after 4th month of pregnancy and the reliability was 91–95%.

Then came the detection of hCG by the Enzyme technique, which proved to be faster and sensitive and specific [8]. Initially, the Polyacrylamide disc gel Electrophoresis was employed by Oya et al., to examine cysteine aminopeptidase(CAP) and leucine aminopeptidase (LAP), which were detected only after fourth month of gestation. In 1973, Oya et al. demonstrated that alkaline phosphatase which is heat stable was detected in pregnancy after fourth month. Only disadvantage was the large quantity of blood required. But this method was reliable and utilized [9].

2.1.3 Placental Messenger RNA (mRNA)

The big breakthrough came with the promising technique of detection of **Placental mRNA** for beta -hCG & hPL by RT-PCR in the bloodstains from pregnant women [10]. A lot of research was done in the 21st century with respect to this.

What is mRNA? mRNA is the step in between protein-encoding DNA translation and the proteins production by ribosomes [11]. Placenta expressed genes based mRNA transcripts easily detected in maternal plasma prove that the source of fetal nucleic acid release is placenta [12]. Only placental trophoblasts express hPL and beta hCG mRNA which is detected in maternal plasma.

Method of Detection of mRNA: In order to detect presence of these mRNA, RT-PCR is the method of choice. Advantages of the RT-PCR technique is manifold. First it is highly specific and sensitive. The second advantage is that it can be designed to be human specific, proving to be advantageous over the immunological tests of pregnancy which have high chances of false positivity [13].

The controversy was that mRNA was thought to be highly unstable and was considered to be degraded quickly making its use as a possible detection tool in old stains questionable. But few studies have refuted this dogma and there are instances wherein the mRNA has been detected in bloodstains as old as 16 years [14].

An ideal mRNA-based test should be in detectable quantities early in pregnancy as well as throughout pregnancy with rapid clearance after delivery. Out of the known 11 genes which were reported to have pregnancy-specific expression patterns, only 2 genes have been thoroughly studied - β-subunit of the hCG and human placental lactogen [13]. Let us look at both the molecules closely to determine which is ideal.

Human Placental Lactogen: The various parts of the hPL mRNA is depicted in **Figure 1**. As per the demonstration, RT-PCR detection of hPL was noted throughout pregnancy increasing until delivery and it was undetectable within 24 hours following delivery, which makes it an ideal test [13]. The added advantage is that, when there is a mixed sample, it can be designed to be human specific [10].

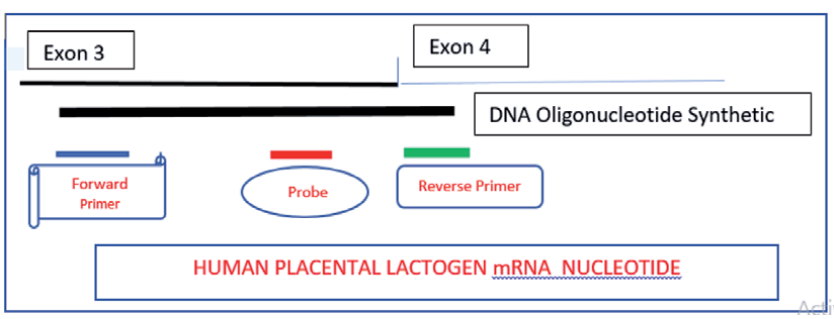

Figure 1.
Human placental lactogen mRNA nucleotide (source: [15]).

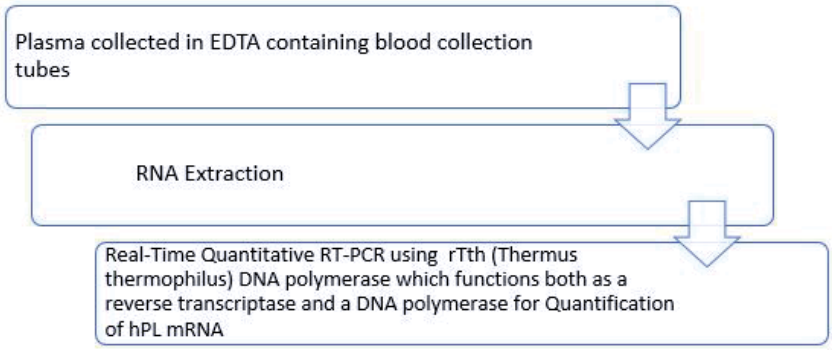

Figure 2.
Method describing the quantification of mRNA using RT PCR technique.

Method of RT PCR for quantification of mRNA [15]:

As in **Figure 2**, the exact methodology of quantification of mRNA using RT PCR technique is described.

Advantages of hPL mRNA: A study done by Gauvin et al. demonstrated the detection of transcripts of hPL in as little as 0.25 cm2 of dried bloodstain, showing that this test has a high sensitivity. The other advantage noted was that the hPL transcript was demonstrated in bloodstains as old as 56 days at room temperature. All these factors- stability and high sensitivity imply that RT-PCR hPL mRNA assay is an ideal marker for pregnancy related forensic diagnostics [10].

β-subunit of the hCG: With respect to the other marker – βhCG, it was noticed that the mRNA concentration reduced as pregnancy advances [16]. The RT-PCR of βhCG mRNA levels are detected in the first trimester where their levels are the highest. After which, the levels decreases as pregnancy advances making its detection difficult. So to conclude, by itself βhCG mRNA may not be reliable as a biomarker when you have to consider the entire duration of pregnancy [13].

Also research has been done in the area to estimate the gestational age from the bloodstains. A study based on the rationale that the use of time-wise reverse expression intensity pattern of the hPL and βhCG transcripts could predict the period of gestation from the pregnant woman's bloodstains. Gauvin et al. tested this hypothesis and found that there was a significant positive relation in women with gestational ages between 8 and 20 weeks. But the biggest disadvantage faced was that the RT-PCR assay for βhCG is less sensitive when compared to hPL.

3. Obstetric markers in substance abuse

3.1 Obstetric markers in alcohol abuse

Alcohol abuse in the mother is a problem which affects the fetus drastically. The teratogenic effects of alcohol on the fetus are well known causing Fetal Alcohol Syndrome, a severe form of affection of the Fetal Alcohol Spectrum Disorder. Also the pregnant woman can commit crimes under the influence of alcohol.

The biomarkers specific to alcohol abuse in pregnancy are ethyl glucuronide (EtG), fatty acid ethyl esters (FAEEs) and ethyl sulphate (EtS) which are non-oxidative direct ethanol metabolites. These remain positive in maternal serum and urine for FAEEs for up to 24 h in serum and EtG in urine up to 5 days. These are promising. Also carbohydrate-deficient transferring (CDT) and phosphatidylethanol in blood of the mother are being evaluated.

3.2 Obstetric markers In drug abuse

Drug abuse in pregnancy is not uncommon. But unfortunately, the teratogenic effect of drugs can prove disastrous on the fetus. Use of cocaine prenatally causes preterm labour, abruption, congenital anomalies and low birth weight babies. Cannabis metabolites use causes difficulty in memory and learning. Opiate exposure prenatally results in withdrawal symptoms in neonates. Evaluation of these drugs in hair of the mother and meconium of the neonate can be done by standard chromatography methods. Information based on maternal hair depends on its length, while the exposure during pregnancy results in these drugs getting accumulated in the meconium, which needs to be analyzed as soon as its passed once fetus is born [17]. The forensic implications are that these pregnant women on drug use exhibit depression, anxiety, psychological struggle, can commit crimes under the drug influence and are liable for arrest and can be tried in the court according to the prevailing laws of the countries where they are prosecuted [18].

4. Obstetric markers in paternity suits

Paternity testing in the prenatal period could be required in cases of pregnancy resulting from rape. Earlier, the paternity testing depended on invasive procedures such as chorionic villus sampling and amniocentesis. These procedures could pose a risk to the wellbeing of the mother and fetus. In order to find a relatively safer testing method, non-invasive methods using the cell free fetal DNA (cffDNA) were investigated [19].

Initially the genetic markers investigated were short tandem repeat (STR) loci. But, the increased stutter amount hindering allelic assignments and decreased size of DNA fragments- maternal & fetal proved to be a hindrance [20]. Also only Y-chromosome STRs (Y-STRs) could be used which restricted the application to male fetuses only and not in female fetuses. The chances of false paternity exclusions were increased [21].

Then the Single nucleotide polymorphisms (SNPs) were investigated. A recent study done by Tam et al. developed a systematic SNPs selection procedure which reduced the number of target-SNPs for sequencing analysis to an average of 148 effective SNPs to calculate the probability of paternity. But the possible drawback is that in order to perform the test, a large number of loci is required [22]. But this is the mainstay for noninvasive paternity testing as of now.

With the number of SNPs to be tested on an average being 148, it can be cumbersome. So research is on to find a better genetic marker. Hence, the use of microhaplotypes is being researched. Microhaplotypes are the regions of ~200 bp containing two or more SNPs and at least three different haplotypes. Microhaplotypes with only 15 regions and with admixtures of DNA are being researched to determine paternity in a non-invasive manner [23].

5. Medicolegal implications

Obstetric markers is emerging as an important aspect in the forensic diagnostics. The pregnant women may face crimes like rape, physical harm against them. While, they may undergo criminal abortions, illegal feticide or have a substance abuse issue for which they can be held liable. The various obstetric markers- use of mRNA in bloodstains, use of biomarkers in substance abuse or the noninvasive genetic

markers for paternity testing, play an important role in solving the investigations and cases. Amongst the tests performed to determine if the bloodstain is that of a pregnant woman, the detection of mRNA of hPL is superior to other DNA analysis methods. Amongst the non-invasive paternity testing methods which is gaining prominence, the SNP detection is far superior to the STR detection. The emergence of use of Microhaplotypes which would further simplify the paternity detection is still under research.

6. Future research avenues

Obstetric markers in forensic diagnostics is an area of potential research. The possible areas would be to identify newer biomarkers which can be detected in bloodstains for a longer duration. Another potential area is to discover a technique which could estimate the gestational age from the blood stains obtained.

7. Conclusions

The field of forensic diagnostics has a widespread implication on solving cases related to pregnancy using specific obstetric markers. These markers are based on molecular technology. Bloodstains of pregnant women are best detected by RT PCR quantification of mRNA hPL. The ideal biomarkers for maternal alcohol abuse is still under investigation. Noninvasive Prenatal diagnostics is a helpful diagnostic aid as it does not harm the mother or fetus. Presently, SNP targets are being used for paternity detection and microhaplotypes as biomarkers are being investigated.

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

mRNA	Messenger ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
PS-beta-G, TSG, PAPP-C (SP 1)	Schwangerschaftspezifisches beta-1-Glykoprotein
hCG	Human chorionic gonadotropin
EDTA	Ethylenediamine tetraacetic acid
cffDNA	Cell-free foetal DNA
STR	Short tandem repeat
SNPs	Single nucleotide polymorphisms

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Section 4

Forensic Medicine

Chapter 9

Salivary Analysis for Medico-Legal and Forensic Toxicological Purposes

Roberto Scendoni

Abstract

Saliva testing has attracted great interest in the forensic scientific landscape recently, especially among institutions or legal authorities interested in determining drug concentrations (for application in the workplace, drug driving, legal issues associated with drug testing, and pharmacokinetics of selected drugs). Indeed, it has been established that oral fluid is an adequate alternative biological matrix to blood for the determination of xenobiotics and/or drugs of abuse and/or metabolites both in living and deceased individuals. The concentration of a detectable substance in saliva is generally proportional to the free fraction of the drug present in plasma; this measurement therefore makes it possible to correlate the concentration of the substance and its pharmacological effects on the individual. The purpose of this chapter is to examine the main analytical techniques developed thus far in saliva drug testing, from screening to confirmatory analysis, taking into account the interpretation of cut-off levels. Both well-defined and potentially problematic issues are highlighted from medico-legal and toxicological perspectives.

Keywords: salivary analysis, drugs, analytical techniques, legal medicine, forensic toxicology

1. Introduction

Detecting the presence of drugs or their metabolites in biological material requires different approaches and methods, depending on the purpose of the investigation and specific legal requirements. In the forensic toxicology field, multiple biological matrices are commonly used as diagnostic tools (such as blood, urine, keratin matrices, oral fluid, etc.) and the respective results, either alone or in combination with each other, provide useful elements for a correct diagnosis. An investigation may be prompted by various concerns: suitability to drive, professional driver suitability, employee and work suitability, suitability for gun permit, suitability for specific competition and/or contractual rules, diagnosis of use/abuse (also in the contexts of custody of minors and international adoptions), diagnosis of drug addiction, and diagnosis of intoxication in living or dead people.

Technical choices are based on these premises and purposes. For example, urine testing can typically determine the "recent" consumption of substances of abuse (with a temporal detection window of hours or even days depending on the pharmacokinetic characteristics of the substance in question). This sample can also

be used to determine chronic drug use if the analysis is extended to several samples collected on different days and "by surprise" (i.e. with the shortest possible notice given to the interested party, not exceeding 24 hours). Chronic use, as well as previous patterns of use/abuse, can be verified by analysis of the hair matrix too.

In cases where it is necessary to quickly evaluate degree of substance intoxication (for example in an emergency situation) blood testing is particularly useful. Even so, over the past few years oral fluid has been increasingly studied as an alternative matrix of choice, and a number of reviews and papers have recently focused on various aspects of drug testing using oral fluid, although it has a shorter detection window than blood (**Figure 1**). Consideration should be given to the importance of oral fluid as a clinical diagnostic [1] and forensic tool and its relevance for a range of applications including workplace drug testing [2], drug driving [3], legal issues associated with drug testing [4], pharmacokinetics of selected drugs [5], and therapeutic drug monitoring (TDM) [6].

Regarding its composition, saliva is a very dilute fluid. Its major constituent is water (> 97%); other components include electrolytes, immunoglobins, enzymes and proteins. In normal conditions, healthy adults produce approximately 500–1500 mL saliva in 24 hours through the submandibular gland (about 65%), the parotid gland (23%) and the sublingual gland (4%), along with many other small glands distributed in the oral cavity (about 8%). Products of the salivary glands can be classified into four major components with different functions: mucus that serves as a lubricant; amylase, an enzyme that initiates the digestion of starch; lingual lipase, an enzyme that begins the fat digestion process; and a slightly alkaline electrolyte solution that moistens food so that it can be swallowed easily.

The most abundant salivary electrolytes are sodium, potassium, chloride and bicarbonate, while calcium, magnesium and phosphate are present in lesser concentrations. Other salivary constituents include substances transported from the blood through the gland into saliva [7].

Salivation can be stimulated or reduced by several factors. Electrolyte concentrations and volume of saliva produced are influenced by the time of day and type of salivation stimulus. In fact, the volume and composition of oral fluid can vary during the day and over time in each individual. Therefore, it can be said that its composition varies continuously, both quantatively and qualitatively [8]. When salivary constituents need to be identified, it should be emphasised that the results will depend on the subject's cooperation, psychological status, medication use, method of sampling and time of day.

Saliva has a slightly more acidic pH (6 to 7) than that of blood, and therefore all lipophilic psychoactive substances, with a weak basic nature, low molecular weight and blood protein binding of less than 50%, are preferentially excreted in saliva by passive diffusion of the free fraction of the substance in its ionised form. Moreover, the pH of saliva can change from being slightly acidic at rest, to basic (pH 8) at

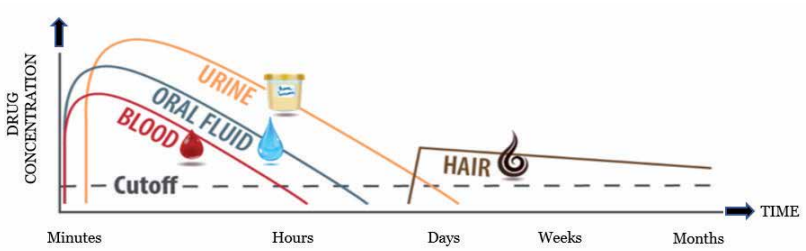

Figure 1.
Drug detection times in different matrices.

ultimate stimulation. Amylase and mucus also increase in concentration after stimulation [8].

The first guidelines for the analysis of substances of abuse in saliva were proposed in 2004 in the United States by the "Substance Abuse and Mental Health Service Administration" (SAMHSA) [9] and were mainly intended for analyses carried out in the workplace to determine the possible use of substances. Subsequently, the "European Workplace Drug Testing Society" (EWDTS) [10] also drafted European guidelines, again oriented to analyses in workplaces. SAMHSA published its final Mandatory Guidelines for Federal Workplace Drug Testing Programs using Oral Fluid on October 25, 2019 in the Federal Register [11]. The new regulations only apply to federal workplaces, at the time of writing, but the impact is sure to reach beyond the initial scope of these regulations.

2. Drug transfer from blood to saliva

The most common routes for a drug to migrate to saliva are passive transcellular diffusion, ultrafiltration, active transport and passive diffusion.

a. **Passive transcellular diffusion:** highly lipid-soluble substances may pass through the capillary wall, basement membrane and acinar cell of the secretory end-piece, with the lipid layer of the epithelial cell wall providing the rate-limiting barrier. The same mechanism would probably enable these molecules to pass through the cells lining the ducts of the gland. The salivary concentrations of the lipid-soluble, unconjugated steroids such as oestriol, cortisol and testosterone approximate the unbound plasma concentrations. But, the concentration of the lipid-insoluble, conjugated steroid dehydroepiandrosterone sulphate is approximately 1% of the unbound plasma concentration [12].

b. **Ultrafiltration (or paracellular transport)**: small polar molecules such as glycerol and sucrose enter saliva. The saliva/plasma (S/P) ratios of several small polar, lipid-insoluble compounds are plotted as a function of their molecular weight (MW). This mechanism is restricted to compounds with a MW of less than about 300 Da, and even those with a MW of about 150 Da are only filtered to a minimal extent. Furthermore, the flow rate of saliva should not affect S/P ratios if diffusion is rapid and passive.

c. **Active transport mechanism:** clearly operates for many electrolytes and for some proteins such as IgA. This mechanism has also been proven for some drugs. Lithium (MW = 7 Da) would be expected to appear in saliva by ultrafiltration. However, the findings of a S/P ratio of more than two indicates an active secretory mechanism [13]. Borzelleca (1965) [14] investigated whether penicillin and tetracycline were secreted in saliva. The secretion of these antibiotics in saliva appeared to be dependent on the concentration in the blood. Since the secretion of penicillin by the salivary apparatus and by the kidney were both inhibited by probenecid, an inhibitor of the active renal pathway, at least a part of the penicillin secretion in saliva involved an active mechanism.

d. **Passive diffusion process**: is characterised by the transfer of drug molecules down a concentration gradient with no expenditure of energy. The rate of diffusion of a drug is a function of the concentration gradient, the surface area over which the transfer occurs, the thickness of the membrane, and a diffusion

RELATING TO DRUG
Lipid-solubility
Acidic or basic, and the pKa
Molecular weight and spatial configuration
Charged or neutral
RELATING TO SALIVA
Saliva pH
Saliva flow rate
Saliva-binding proteins - usually minimal
Enzymes in saliva capable of metabolising the drug
RELATING TO THE CIRCULATING DRUG LEVEL IN THE FREE (NONPROTEIN-BOUND) FORM
Dose and clearance of drug
Nonprotein-bound blood level

Table 1.
Factors influencing passive diffusion of a drug from blood to saliva.

constant that depends on the physico-chemical properties of each drug [15]. The variables which influence this type of transport are listed in **Table 1** (Landon and Mahmod, 1982) [16].

Salivary secretion is a reflex response controlled by both parasympathetic and sympathetic secretomotor nerves. This is an important factor influencing oral fluid availability and potential drug concentrations. Taking medication which affects either the central nervous system or the peripheral nervous system (or medication which mimics the latter as a side effect) alters salivary composition and salivary volume. Therefore, patients suffering from systemic diseases may show alterations in salivary gland secretion and electrolyte concentrations. Finally, diet and age also have an impact on composition and volume of saliva [8].

3. Methods and techniques

3.1 The sampling

It is essential to prepare Standard Operating Procedures (SOPs) relating to the collection and storage of the oral fluid sample, as well as the training of personnel assigned to take and ship the sample to the laboratory where the toxicological analysis will be carried out. It follows that it is essential to document:

- respect for the privacy and security of the person undergoing analytical assessment;
- the identity of the person undergoing analytical assessment;
- the location where the sample of oral fluid has been collected;
- that no falsification or tampering of the sample has taken place;

- that the informed consent form has been completed in its entirety by the person undergoing the analytical assessment (unless there is a formal mandate from the Legal Authority);

- the use of particular medicines that may interfere with the analytical results;

- the traceability of the sample through appropriate records of its movement, from the place of sampling to the laboratory that receives it, including the identity records of the personnel authorised to handle it.

Neat oral fluid can be collected from expectoration (or spitting), but this is relatively viscous and can therefore be challenging to work with and analyse in the laboratory. It may also be contaminated with food and oral debris, which makes centrifugation essential. In addition, sensitive detection techniques are required, because the volume collected will often be less than 1 mL. Normally, the absorbent foam swab or pad used to collect the oral fluid is added to a diluent. After mixing, the solution is ready for drug analysis. Other devices involve squeezing absorbed oral fluid from a pad or foam directly onto the drug-detection device, a process that can take one to three minutes. A number of devices incorporate some form of indicator to show when an adequate amount of oral fluid has been collected [17].

A number of drugs affect the secretion of oral fluid [8], mostly cannabis and amphetamines, including designer drugs such as MDMA. Other drugs include the sedating antihistamines, antipsychotic drugs, anticholinergic drugs and several antidepressants. Less commonly used drugs increase saliva flow and these include clonidine, pilocarpine and beta-2 stimulants (salbutamol, terbutaline, etc). Overall, there is significant intra- and inter-subject variation in relation to drug concentrations depending on the technique used, the physiology of the person and the factors affecting drug concentration in oral fluid.

3.2 Analytical techniques

An important aspect to consider is the choice of analytical technique used for the detection of drugs and metabolites in saliva. A fundamental element is the certainty and reliability of the results, from both qualitative and quantitative perspectives. The results of quantitative determination, though, are not easy to interpret as the information that makes it possible to trace the metabolic process is often unavailable (e.g. time the drug was taken, amount of active ingredient, and route of administration).

In many forensic contexts, oral fluid is analysed with screening methods. The semiquantitative results obtained must be validated by confirmatory techniques, such as liquid chromatography combined with mass spectrometry [18]. Oral fluids (OF) have been recently introduced as a biological matrix useful for roadside testing to determine illicit drug use because the time course of drugs in oral fluid may resemble that of plasma. Moreover, OF can be considered a valid alternative specimen for confirmation testing because drugs are excreted in saliva mainly as parent compounds [19–21]. In fact police officers, without medical supervision, are not authorised to employ invasive methods but they can collect OF samples. A very comprehensive review of the analysis of drugs of abuse in OF was conducted by Reinstadler et al. [22]. Other studies [23] have highlighted the importance of both the sample treatment process and the use of hyphenated instruments in obtaining analytical performances that satisfy current regulations in terms of sensitivity, selectivity and fast confirmatory analysis.

3.2.1 On-site screening test

Recent data have shown improvements in the effectiveness of on-site drug testing using oral fluid, and significant progress has been made in terms of sample collection and accuracy of analysis [24].

A number of field drug testing devices are available and used in many countries to perform on-site testing on oral fluids in the context of Driving Under the Influence of Drugs (DUID) [25]. For example, DrugWipe® is an immunochromatographic test strip, based on the Frontline urine test strip from Boehringer Mannheim. A pink colour in the test window indicates the presence of the analyte in question, but different devices are normally required to detect the various classes of drugs of abuse. However, a recent version of this device, DrugWipe 5A, is capable of indicating the simultaneous use of cannabis, amphetamine, methamphetamine, ecstasy, cocaine, and opiates [26]. A recent study investigated the reliability of DrugWipe 5A in establishing exposure to principal drugs of abuse (cannabis, amphetamines, cocaine, and opiates) using oral fluid specimens by comparing the on-site results with headspace solid-phase microextraction (HS-SPME) gas chromatography–mass spectrometry (GC–MS) analyses on extractions from the sample collection pad [27].

Another point of collection test, Rapid STAT®, has broken new ground by combining the convenience of oral fluid collection, surface wipe testing or pure substance measurements with the sensitivity, accuracy and precision of a laboratory based test, with speedy results (in a few minutes).

Table 2 shows the recommended minimum detectable concentrations of drugs in oral fluid according to SAMHSA and European Union roadside assessment testing study (ROSITA) cut-off levels [28].

3.2.2 Laboratory screening test

The enzyme-linked immunosorbent assay (ELISA) is a sensitive and versatile test used in many fields to detect and measure substances in biological samples (**Figure 2**). For almost 50 years it has remained a trusted testing technique for everything from food allergen detection to medical screening for various illnesses. For the toxicology market specifically, ELISA is an excellent and cost-effective solution which meets high-throughput screening (HTS) needs. The procedure is simple and easily automated or it can be conducted by a laboratory technician. It basically works around the principle of competition between two substances in a given sample: an enzyme conjugate such as horseradish peroxidase (HRP) is used to compete with a target substance for a limited number of specific binding sites on a precoated microplate.

Drug	*SAMHSA cut-offs (ng/mL)*	*ROSITA cut-offs (ng/mL)*
Cocaine	00	5–10
Morphine	40	—
6-AM	4	10
Methamphetamine/Amphetamine/MDMA	50	70–90
THC	CM	1.9

6-AM = 6-Acetylmorphine, MDMA = methylenedioxymethamphetamine, THC = Δ^9-tetrahydrocannabinol.

Table 2.
Recommended minimum detectable concentrations of drugs in oral fluid – Instrumental devices field testing.

Figure 2.
Fully automated Elisa analyser.

The different available types of ELISAs provide a reliable means for screening oral fluid. In general these work adequately for amphetamines [29], buprenorphine, cocaine [30], methadone [31], and other opioids [32]. Cannabis may pose more difficulties, particularly if the immunoassay has little cross-reactivity to *tetrahydrocannabinol* (THC), the main psychoactive component of the drug. Even so, enzyme immunoassay has been successfully used for cannabis; the same applies to benzodiazepines despite their low oral fluid concentrations [33].

3.2.3 Confirmatory analysis

Confirmatory techniques for drugs in oral fluid [20] are mostly adapted from those used in the analyses of blood or plasma/serum specimens. Recovery of drugs is not typically a limiting factor, considering the higher water content and lower protein levels of oral fluid compared to blood. However, the sample volume of oral fluid will be smaller, with potentially lower concentrations, which means that more adjustments are required to analytical techniques. Indeed, in saliva analysis the detection or quantification limit for drugs is very much determined by the type of screening test and its application. The confirmation method must be able to produce an analytical result that is optimally independent from that of the screening. Therefore, it must be based on different physico-chemical principles and have superior analytical selectivity and sensitivity. In this regard, a quantitative confirmatory method capable of reaching a lower limit of quantification (LLOQ) equal to at least half the cut-off of the screening method is considered acceptable. The use of a confirmatory method which is based on the measurement of a similar analytical signal is not acceptable since it is highly correlated to that of the screening (e.g. confirmation of a given immunochemical with another immunochemical method). The use of an identical chromatographic technique to confirm a set of data obtained by chromatography is acceptable if the detection technique combined with chromatography changes.

The use of a chromatographic technique to confirm screening data obtained by chromatography with the same detection system is allowed only if the two separation techniques produce poorly correlated results (for example, two series of significantly different retention times, with the use of columns of different polarity or selectivity, etc). However, in the forensic toxicological field, chromatographic separation is always necessary in a confirmatory method; the general consensus of the international scientific community is that mass spectrometry (MS) with its many methodological possibilities can be combined with a chromatographic separation technique such as gas chromatography (GC), high pressure liquid chromatography (HPLC) or capillary electrophoresis (EC) for confirmatory analysis (**Figure 3**). Many methods

Figure 3.
Ultra-high performance liquid chromatography.

use LC–MS as distinct from GC–MS to cater for the lower sample volumes and low detection limits, although a number of GC–MS techniques have exhibited adequate sensitivity [34].

4. Medico-legal and toxicological issues

4.1 Saliva versus blood

Intra-individual variability of the S/P ratio has been demonstrated for a number of drugs administered orally or intravenously [35]. Following the uptake of an orally applied substance in the intestine, arterial blood has a higher concentration than venous blood (positive arteriovenous difference). If the substance is completely absorbed but not significantly metabolised in a particular organ, the situation is reversed: the substance rediffuses from the cells into the blood (negative arteriovenous difference in the elimination phase). The various organs can be classified into two groups: those with a high blood flow (e.g. liver, kidney, brain, salivary glands), and those with a relatively low blood flow (e.g. skin, resting skeletal muscle, fat). In pharmacokinetics the first group of highly perfused organs is included in the central compartment, while the second group of less perfused organs belongs to the peripheral compartment. This must be taken into account when saliva concentrations are compared with blood concentrations from cubital veins in the peripheral compartment. In any case, salivary glands have a high blood flow, which means that the arteriovenous difference of freely diffusible substances is relatively small, with a ratio close to 1.0. Poor correlations between the two compartments have been documented in the literature, but neglect of the phenomenon described can only partly account for this [36].

Some comparative studies [37, 38] have found that drug concentrations in oral fluid cannot be used to accurately estimate drug concentrations in blood. A positive result in an oral fluid test may certainly confirm recent drug use, but it may only provide a semiquantitative assessment of the drug concentration in the blood (and only for some drugs). For psychiatric patients, oral fluid testing may be used as a non-invasive technique for evaluating substance use. In the case of drivers suspected of driving under the influence of drugs, oral fluid may be used for initial on-site screening tests (afterwards, it may be decided that a blood sample should be taken for forensic drug analysis).

Wille et al. (2009) [37] analysed blood and saliva samples by gas chromatography–mass spectrometry (GC–MS) or liquid chromatography-mass spectrometry (LC–MS). Scatter plots and trend lines of the blood and oral fluid concentrations were created and the median, mean, range, and standard deviation (SD) of the oral fluid to blood (OF/B) ratios were calculated for different classes of drugs, including amphetamines, benzodiazepines, cocaine, opiates, and delta^{9}-2 tetrahydrocannabinol. The ratios found in this study were in line with previously published results, but the range was wider. The OF/B ratios of drugs of abuse such as amphetamines, cocaine, and opiates were > 1 [amphetamine: median (range) 13 (0.5-182), methylenedioxyamphetamine: 4 (1-15), methylenedioxymethamphetamine: 6 (0.9-88), methamphetamine: 5 (2-23), cocaine: 22 (4-119), benzoylecgonine: 1 (0.2-11), morphine: 2 (0.8-6), and codeine: 10 (0.8-39)]. Unsurprisingly, the ratios for benzodiazepines were considerably lower: given their high protein binding and weak acidity, benzodiazepines typically have low oral fluid concentrations [diazepam: 0.02 (0.01-0.15), nordiazepam: 0.04 (0.01-0.23), oxazepam: 0.05 (0.03-0.14), and temazepam: 0.1 (0.06-0.54)]. For tetrahydrocannabinol, an OF/B ratio of 15 was found (range 0.01-569). The variability of the OF/B ratios in suspected drugged drivers was clearly mirrored in the data. Be that as it may, blood concentrations could not be reliably calculated from oral fluid concentrations, due to the wide range of ratios.

Gjerde H et al. (2010) [38] analysed 90 pairs of blood and oral fluid specimens from patients undergoing acute psychiatric treatment and 22 pairs of blood and oral fluid specimens from suspected drugged drivers, with the aim of comparing drug concentrations between the two biological matrices. The median oral fluid/blood drug concentration ratios for the most prevalent drugs were 0.036 diazepam, 0.027 nordiazepam, 7.1 amphetamine, 2.9 methamphetamine, 5.4 codeine, 1.9 morphine, and 4.7 tetrahydrocannabinol. For the six most prevalent drugs, the correlation coefficients between drug concentrations in oral fluid and blood ranged from 0.15 to 0.96. The results, therefore, showed large interindividual variations in drug concentration ratios between oral fluid and blood. This wide distribution of OF/B ratios indicated that drug concentrations in oral fluid may not be used to reliably estimate drug concentrations in blood.

Such analytical variability could cause controversy in the judicial field, especially when the values obtained from saliva are only slightly higher than the cut-off levels established by the law of various countries.

Drug	*Average oral fluid to blood concentration ratio*
Barbiturates [39]	0.3
Ethanol [40]	1.07
Buprenorphine [41]	1
Codeine [42]	4
Methamphetamine [43]	2
MDMA [44]	7
Cocaine [45]	3
Diazepam [46]	0.01–0.02
Methadone [47]	1.6
Morphine [48]	0.8
Δ9- Tetrahydrocannabinol [49]	1.2

Table 3.
Average oral fluid to blood concentration ratios for selected drugs.

Table 3 shows the average values of oral fluid to blood concentration ratios of selected drugs, based on various pharmacokinetic studies; the average ratios change depending on a number of factors, such as pH of oral fluid, protein binding and degree of contamination of the membranes in the oral cavity by recently consumed drug.

4.2 Cut-off levels and analytical interpretation

Interpretation of oral fluid drug test results depends to some extent on the purpose of testing. An employer may decide to implement a workplace drug testing programme primarily to detect drug abuse among employees (or even job applicants), especially regarding safety-sensitive positions or following a safety incident or accident. Random workplace testing could also serve as a deterrent to substance misuse in the general workforce. Drug treatment specialists carry out drug testing to foster drug abstinence and compliance with programme requirements. Numerous factors must be considered when interpreting drug test results. During this process, complex questions may be posed, depending on the nature of the drug-testing programme, and sometimes the answers sought go beyond reasonable scientific certainty. Patterns of metabolic disposition should be understood for each class of drugs. Of course, the interpretation of oral fluid tests requires knowledge of the unique features of this biological matrix, along with a thorough understanding of: the chemical and physiological factors that affect drug transfer into oral fluid; analytical factors; kinetic aspects of drug disposition; drug metabolic patterns; and potential risks of oral contamination and passive exposure. Generally speaking, it has been shown that oral fluid tests are most useful in the detection of recent drug use [50].

The use of a screening method can be justified in a forensic toxicology laboratory when there is a need to analyse a large number of samples in a short time and at low costs, with the advantages of high or total automation. Screening methods usually employ colorimetric, enzymatic, and immunochemical techniques. However, screening methods are characterised by low specificity (qualitative data) and high inaccuracy (quantitative data), particularly when several chemical species can be detected in the sample but not discriminated by the method (e.g. an unchanged compound and its metabolites, or various types of similar species of compounds). Given their intrinsic characteristics, these methods exclusively produce a presumptive result, that is to say the probable negativity (absence) or positivity (presence, better defined as "non-negativity") of the sample with respect to an analyte, or more often a class of substances, relative to a cut-off value set by the method. In any case, whatever the analytical specificity of the screening method, a positive result obtained through a single screening test cannot have forensic validity. It is therefore essential that this result is verified by a confirmatory analysis on a new sample rate.

The results of a quantitative analysis must be expressed in a uniform unit of measurement, so as to exclude interpretative doubts, directly comparable with any reference values (cut-off) and accepted by the International System of Units (SI). The uncertainty associated with the measurement performed must be indicated; at the same time, the comparison with threshold or reference values must take into account this uncertainty. **Tables 4** and **5** show the recommended cut-off levels of oral fluid tests according to EWDTS and SAMHSA guidelines.

Drugs and metabolites can be detected for a period of several hours to several days following drug exposure. Their concentrations in oral fluid are generally related to content in blood, but may also be present as residual drug in the oral cavity [11].

In what follows, descriptions of the main drugs of abuse are given [50].

Drug	*Screening Cut-off (ng/mL)*	*Confirmation Cut-off (ng/mL)*
OPIATES		
Morphine	*Opiates (Morphine) 40 Opiates (6-MAM) 4*	*15*
Codeine		*15*
Norcodeina		*2*
6-Acetylcodeine		*2*
Dihydrocodeine		*15*
6-Monoacetylmorphine		*2*
METHADONE AND METABOLITES	*L-Methadone, 50*	20
BUPRENORPHINE AND METABOLITES	5	1
COCAINE AND METABOLITE		
Cocaine	*Cocaine + metabolites 30*	*8*
Benzoylecgonine		*8*
AMPHETAMINE AND CONGENERS		
methamphetamine	Amphetamines 40	*15*
amphetamine		*15*
MDMA		*15*
MDA		*15*
CANNABINOIDS		
THC	*THC 10*	*2*

Table 4.
Recommended maximum screening and confirmation cut-off values for oral fluid tests in the workplace according to EWDTS guidelines [51].

AMPHETAMINE: a synthetic substance related to natural sympathomimetic amines with central nervous stimulant activity. Amphetamine appears rapidly in oral fluid following administration and parallels plasma drug concentrations. Amphetamine is also produced as a metabolite of methamphetamine and from a variety of pharmaceutical products. A positive test result for amphetamine indicates amphetamine use; determination of d/l-isomer ratio should rule out the possibility of mystification with another drug.

METHAMPHETAMINE: a synthetic sympathomimetic amine with central nervous stimulant activity similar to amphetamine but with more lasting effects. It is misused in numerous ways including smoking, snorting, injecting, and oral administration. Methamphetamine and amphetamine appear rapidly in plasma and oral fluid following administration. Determination of d/l-isomer ratio rules out the possibility that methamphetamine presence is due to the metabolism of another drug or use of an over-the-counter nasal inhaler. A positive test result for methamphetamine and amphetamine (methamphetamine < amphetamine) indicates possible combined use of methamphetamine and amphetamine.

METHYLENEDIOXYMETHAMPHETAMINE (MDMA): a synthetic, ring-substituted amphetamine derivative. N-demethylation of MDMA yields 3,4-methylenedioxyamphetamine (MDA), an active metabolite exhibiting similar pharmacological properties as the parent drug. O-demethylenation of MDMA and MDA produces 3,4-dihydroxymethamphetamine (HHMA) and

Initial Test Analyte	*Screening Cut-off (ng/mL)*	*Confirmatory Test Analyte*	*Confirmatory Test Cut-off (ng/mL)*
THC (Cannabis)	4	THC	2
Cocaine/Benzoylecgonine	15	Cocaine/Benzoylecgonine	8
			8
Codeine/Morphine	30	Codeine	15
		Morphine	15
Hydrocodone/Hydromorphone	30		15
		Hydrocodone/Hydromorphone	15
Oxycodone/Oxymorphone	30	Oxycodone/Oxymorphone	15
			15
6-Acetylmorphine (heroin)	4	6-Acetylmorphine	2
Phencyclidine (PCP)	10	Phencyclidine	10
Amphetamine/Methamphetamine	50	Amphetamine/Methamphetamine	25
			25
Methylenedioxymethamphatime (MDMA)	50	MDMA MDA	25
			25
Methylenedioxyamphetamine (MDA)			

Table 5.
Cut-off levels of oral fluid testing according to SAMHSA oral fluid guidelines [11] (effective January 1, 2020).

3,4-dihydroxyamphetamine (HHA), respectively. MDMA is typically administered orally and reaches maximal blood concentrations in approximately 2 hours. Oral fluid concentrations of MDMA are highly correlated with plasma MDMA. Oral fluid concentrations of MDMA are an order of magnitude higher than in plasma; this is attributed to the high pKa of MDMA and low plasma–protein binding. A positive test result for MDMA (no MDA) indicates illicit MDMA use; a positive test result for MDMA and MDA suggests illicit MDMA use (presence of MDA probably due to metabolism of MDMA to MDA but if MDA ≥ MDMA, a combined use of illicit MDMA and illicit MDA is admissible).

3,4-METHYLENEDIOXYAMPHETAMINE (MDA): a synthetic, ring-substituted amphetamine derivative. MDA has been reported to appear in oral fluid following the administration of MDMA in concentrations representing approximately 4–5% of MDMA. Possible sources of MDA: illicit MDA, metabolite of illicit MDMA, metabolite of illicit MDEA. However, the confirmed presence of HHA and/or HMA in oral fluid would be useful to substantiate MDA use.

3,4-METHYLENEDIOXYETHYLAMPHETAMINE (MDEA): a synthetic analogue which is generated when an ethyl group is substituted for the methyl group of MDMA. MDEA is metabolised by O-demethylenation and by N-dealkylation of the ethyl-group. The major metabolite is formed by O-demethylenation to yield N-ethyl-4-hydroxy-3-methoxyamphetamine (HME); N-dealkylation leads to the formation of the active metabolite MDA. A positive test result for MDEA without MDA means illicit MDEA use, otherwise (in co-presence with MDA) combined use or an initiated metabolism of MDEA.

DELTA-9-TETRAHYDROCANNABINOL (THC): a naturally occurring psychoactive constituent of *Cannabis sativa*. THC appears rapidly in plasma following the smoking of cannabis products and is found in oral fluid following smoked and oral ingestion. According to several studies, THC is more highly present in oral fluid than blood, primarily as a result of deposition in the oral cavity. THC tends to decline in a similar manner to plasma concentrations.

COCAINE: a natural stimulant compound made from the leaves of the coca plant. Cocaine has a short half-life (approximately 1 hour) and is rapidly hydrolysed by hepatic esterases to benzoylecgonine (BZE) and ecognine methyl ester (EME). Cocaine and its metabolites appear rapidly in oral fluid following all routes of administration. Cocaine concentrations decrease rapidly within approximately 1 hour; thereafter, oral fluid concentrations appear to decline in parallel with concentrations of the drug in the blood. If cocaine concentration > BZE concentration: cocaine has probably been taken within the past 2–8 hours; cocaine concentration < BZE concentration: cocaine use in the past 12 hours for occasional users and 48 hours for daily users.

HEROIN: a semisynthetic opioid, diacetyl derivative of morphine prepared from opium for the illegal drug trade. Heroin is most commonly administered intravenously and by other parenteral routes, but may also be smoked. Heroin and 6-acetylmorphine appear in oral fluid within 2 minutes of administration. Drug and metabolite concentrations in oral fluid are generally similar to blood concentrations following intravenous administration, but may be substantially higher than blood when smoked. Elevated drug and metabolite concentrations following smoking are probably a consequence of residual drug deposited in the oral cavity. Thirty to sixty minutes after heroin is smoked, concentrations in oral fluid diminish considerably and begin to reflect blood concentrations. If 6-acetylmorphine and morphine are detected, the use of heroin (and not morphine) can be confirmed.

MORPHINE: a natural opiate alkaloid isolated from the plant *Papaver somniferum*; it is also a metabolite of heroin and codeine. Following parenteral administration, morphine appears rapidly in saliva. Cone [48] reported an approximate

45-minute delay in equilibration of morphine concentrations in saliva compared to plasma following intramuscular administration of 10- and 20-mg doses; thereafter, saliva concentrations paralleled plasma concentrations. Morphine can be detected in oral fluid following intravenous administration, the smoking of heroin and poppy seed ingestion. Positive tests for morphine and codeine (with higher codeine concentration) implies codeine use.

CODEINE: a naturally occurring phenanthrene alkaloid and opioid agonist. It appears to be most commonly taken orally. While it is not a metabolite of morphine, it is metabolised by oxidation to morphine and norcodeine and by conjugation. Kim et al. [52] demonstrated that following oral administration of 60 and 120 mg, codeine appeared in oral fluid within an hour and reached maximum concentration in approximately 1.6–1.7 hours. Concentrations in oral fluid correlated significantly with plasma concentration and were three to four times higher in oral fluid than plasma. Codeine could be detected in oral fluid for approximately 21 and 7 hours at cut-off concentrations of 2.5 and 40 ng/mL, respectively. Following intramuscular codeine of 60 and 120 mg, codeine appeared rapidly in oral fluid and reached maximal concentrations in 0.5–0.75 hours. A positive test result for codeine and morphine generally indicates codeine use.

METHADONE: a synthetic opioid used widely as an analgesic as well as in maintenance therapy for persons with opioid dependency. Methadone undergoes extensive metabolism in the liver to form cyclic metabolites, 2-ethylidene-1,5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP), and other minor metabolites. Methadone and EDDP appear rapidly in oral fluid and correlate with plasma concentrations. Therefore, the confirmed presence of oxidative metabolites such as EDDP and EMDP in oral fluid would be useful to substantiate use.

BUPRENORPHINE: an orally available, semisynthetic opioid analgesic, used as a pain reliever and in the management of opioid dependence. Following sublingual administration, buprenorphine reaches maximal plasma concentrations in 1.3–1.6 hours. Its main metabolite is norbuprenorphine. Cone [48] reported measurements of buprenorphine in saliva following intramuscular and sublingual administration of single doses of buprenorphine. Drug concentrations in saliva were substantially lower than plasma at all times following intramuscular administration and were substantially higher following sublingual administration. The low S/P ratio following intramuscular administration is probably due to the high fraction of drug that is protein-bound in plasma. Close correspondence between saliva and plasma buprenorphine concentrations was observed in subjects who administered buprenorphine sublingually on a daily or every-other-day basis. If the oral fluid test reveals buprenorphine ≤ norbuprenorphine, this suggests chronic buprenorphine use.

4.3 Quality assurance of the analysis

Drug testing laboratories must implement a quality management system that includes all aspects of the testing process, such as sample reception, chain of custody, safety and reporting of results, screening and confirmation tests, certification of calibrators and controls, and validation of analytical procedures.

Hence, the laboratory should remain constantly updated on the evolution of analytical techniques, whether for finding new drugs or responding to requests for investigations concerning narcotic and pharmacological drugs. A highly qualified analytical chemical-toxicological laboratory depends on ISO/IEC 17025 [53] accreditation standards and procedures to demonstrate that it operates competently and generates valid results. These standards are agreed by experts the world over,

thus promoting confidence in the work of accredited laboratories and other bodies, on national and international levels, and facilitating cooperation between them. With ISO certification, results are more widely accepted between countries without the need for further testing, consequently improving international trade.

Of course, the benefits of advanced equipment in the chemical-toxicological laboratory go hand in hand with the expertise of qualified personnel with specific competence and adequate scientific training (not restricted to the analytical chemical field). Only through this combination (state-of-the-art tools plus qualified personnel) will it be possible to develop new analytical methodologies in the field of toxicological-forensic analysis and respond to administrative, criminal and social needs imposed by the legal system.

4.3.1 Validation of an analytical method for the detection of drugs in saliva

The validation of analytical methods includes procedures designed to establish that a particular method, used for the identification and/or quantification of an analyte in a given biological matrix, is reliable and reproducible. It is a question of demonstrating that the performance characteristics of the method meet all the requirements for its intended purpose and application. Any analysis methodology used routinely by the laboratory must be previously validated according to internationally agreed procedures [17].

For the most commonly used screening tests, validation procedures are not usually necessary as the method is validated by the manufacturer. In any case, the analysis kit includes calibrators and controls which are to be inserted into each batch of samples to be analysed in order to verify the accuracy and precision of the analyses (according to predetermined target values). In the event that changes are introduced which deviate from the manufacturer's instructions (for example, the biological matrix used is not the one indicated by the manufacturer, variation of the quantification limit, etc.) the laboratory must carry out a complete validation of the method/modified kit. It is best to fully respect the instructions provided by the manufacturer in the use of a kit, or in any case modifications should only be carried out in cases where it is not possible to use other methods.

The analysis methodology can be used routinely by the laboratory if the calculated validation parameters fall within the limits established by the relevant international directives [54].

The use of a good internal quality programme guarantees the reliability of the analytical results and avoids any random errors that may occur in the analytical and/or pre- or post-analytical phase that may affect the accuracy of the result.

The laboratory must participate in appropriate external quality assessment programmes. Analytical performances outside the criteria established by the External Quality Assessment (EQA) programme must be promptly corrected. The choice of one programme over another must be made on the basis of the best scientific evidence obtainable. Participation may concern the identification of classes of substances or individual substances and quantification in the case of confirmatory analyses according to the legal cut-offs or established by the management body of the programme.

In the case of screening tests, the expression of the results is generally in terms of "positive" or "negative". In the case of confirmatory analysis, it is necessary to provide not only qualitative but also quantitative data, namely the concentration detected according to a given calibration curve for the analyte identified in the saliva sample. The results of participation in the EQA are useful for the laboratory director and staff in helping to gauge the performance of the laboratory. In the event of errors, it is important to identify the causes and implement corrective actions that prevent them from recurring.

4.3.2 The activity of the toxicology laboratory for forensic purposes

When the presence of drugs is confirmed in oral fluid, the person under investigation may request the counter-analysis of another aliquot (B) of the saliva sample. This second test can be performed at the same laboratory that analysed the first aliquot (A) of the saliva sample or at another laboratory chosen by the subject in question. Aliquot B must be accompanied by a chain of custody form and include information regarding the results of the original analysis and the cut-offs used. Any laboratory that conducts analysis on aliquot B of the saliva sample must have documentation to demonstrate the use of validated analysis methodologies that meet the precision and accuracy criteria appropriate to the required analyses. It is crucial to guarantee the chain of custody [55], a documented procedure designed to ensure the authenticity, integrity and traceability of a sample from the moment of collection to its disposal. Following the proper chain of custody protocol is fundamental in the reconstruction process and ensures that the sample can be located at any point, unequivocally identified, stored correctly under the right conditions, and protected from tampering and voluntary or involuntary adulterations in all phases. Documentation of the chain of custody must also record every movement and manipulation of the sample, on which dates and under whose care. In the judicial field, the chain of custody is deemed broken in any of the following scenarios (these shortcomings will lead to dispute and may even constitte instances of mystification):

- Missing or non-identical barcodes.
- Missing documentation (supposed to be attached).
- Absence of the informed consent of the person subjected to analytical assessment.
- Broken or tampered safety seals on sample containers or transport container.
- Absence of security seals.
- Insufficient sample volume for testing.
- Containers not intact and evident loss of sample.

On the basis of what has been said thus far, it is evident that laboratory staff are required to fulfil many responsibilities, with potential repercussions in the forensic field in the event of proven professional malpractice. It is therefore necessary to:

- define the type of services that can be provided (screening analysis and confirmation analysis), the suitability of resources and the guaranteed level of safety and reliability;
- ensure the availability of sufficient, adequately trained staff with the necessary experience to monitor and conduct the required laboratory tests (specifically, the analysis of substances of abuse on saliva samples);
- assure the competence of laboratory staff, document in-service training, validate the analytical method, and re-evaluate work performance;

- provide the personnel of the laboratory with access to the complete, updated Standard Operating Procedures (SOP) manual;
- maintain an internal quality control programme which ensures that the analyses are performed correctly and that the results of the tests are communicated in compliance with SOPs;
- participate in appropriate External Quality Assessment (EQA) schemes;
- maintain acceptable analytical performance for all analysis methodologies applied in the laboratory;
- guarantee and document the validity, reliability, accuracy, precision and performance characteristics of each analysis and each analysis system;
- ensure that the necessary corrective actions are taken to maintain laboratory operation and performance at satisfactory levels (e.g. when a quality control system indicates non-compliance with performance specifications, or in response to errors in reporting results or in the analysis of the results of an EQA); the analytical results must not be reported until all the appropriate corrective actions have been taken.

In this context, the forensic toxicologist clearly has a fundamental role, with the responsibility of interpreting the analytical results of tests for substances of abuse in oral fluid at the request of a Legal Authority, competent doctor, potential customer or designated expert representative.

5. Conclusion

Oral fluid testing for drugs of abuse offers significant advantages. A saliva sample can be collected under direct observation with reduced risk of adulteration and substitution and in a less embarrassing or unpleasant manner than urine or blood collection. As oral fluid collection is non-invasive, most people find the procedure more acceptable than having to provide other biological matrices, and suitable hygiene conditions can be respected while the donor is under the collector's observation.

By providing an estimate of the actual circulating amount, the measurement of a drug concentration in oral fluid can be used for the determination of intoxication. In fact, measurements of oral fluid drug concentrations will usually be of value only if they accurately reflect the plasma level. Therefore, before designing a useful model for the salivary secretion of drugs, it is necessary to constantly update information about the relationship between the saliva concentration level of each drug and its plasma concentration level, the mechanisms by which drugs enter oral fluid, and also the effect on salivary flow rate, production in the salivary glands, and the nature of any protein binding in the saliva.

It is very useful to know the limitations and possibilities of salivary analysis in forensic and diagnostic fields. Standardisation of the conditions for collection of oral fluid is strictly essential for achieving reliability and interpretation of the data. Furthermore, appropriate cut-off concentrations need to be established in the development of guidelines for oral fluid testing [56]. In future research, the mechanisms by which drugs enter the saliva must be clarified more adequately.

These considerations are matters of ongoing discussion in the scientific community, in particular the proposed initial screening and confirmatory cut-offs.

When an oral fluid test is performed on a corpse, the forensic pathologist must be accompanied by a toxicologist for the interpretation of the analytical data. It should also be noted that significant ethical issues are involved in the study of many licit and illicit drugs that preclude or limit the study of their short- and long-term effects under "real-world use" conditions, which means that some knowledge will always remain inaccessible.

Finally, there are some open questions and limitations to consider in salivary analysis for forensic purposes. Despite a substantial number of clinical studies on drug disposition in oral fluid, many psychoactive drugs have not been studied. Benzodiazepines and barbiturates and some opioid products have received limited or no evaluation in oral fluid; meanwhile, there is a lack of controlled dosing studies of hallucinogens in humans. Furthermore, the dynamic nature of oral fluid, especially its pH, can substantially affect drug concentrations of basic drugs. It follows that, to date, in a forensic context, the result of an oral fluid test remains questionable, not only for reasons strictly connected to pharmacokinetic and metabolic characteristics, but also for purely analytical reasons:

a. difficulty in applying standardised procedures for sampling;

b. frequent smallness of the sample compared to conventional matrices (e.g. blood) with consequent limitations in terms of multiclass analyses and sampling for counter-analyses;

c. variability of the relationship between salivary and blood concentrations as a function of the variability of salivary pH;

d. possibility of contamination of the oral cavity after ingestion of a substance via intranasal use or inhalation.

e. laboratory deficiencies and/or incorrect application of the analytical procedures.

Therefore, salivary analysis for forensic purposes, now and in the future, necessarily requires a union between highly qualified personnel (able to apply analytical methods and interpret results in the light of up-to-date scientific knowledge) and toxicological laboratories equipped with state-of-the-art instrumentation.

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Chapter 10

Pharmacogenetics and Tramadol-Related Fatalities

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Abstract

Tramadol (TR) is a widely prescribed pain killer because of its relatively safe profile among opioids. Nevertheless, intoxication can occur and overdose can lead to fatal outcomes. Surprisingly, in some fatalities for which death is attributable to TR alone, *postmortem* blood concentration levels overlap with the therapeutic concentration range. These fatal cases might be explained by pharmacokinetic and pharmacodynamic properties of TR that are known to be both enantioselective and influenced by genes. Indeed pharmacogenetics (PG) is of great importance in this issue as it has the ability to elucidate the genetic variation contributing to drug absorption, distribution, metabolism, excretion, and response so that adverse drug reactions, toxicity, and even death can be avoided. The aim of this chapter is to present this issue.

Keywords: tramadol, pharmacogenetics, toxicology, *post-mortem* investigation, molecular autopsy

1. Introduction

There is large interindividual variability in drug response and toxicity, as well as in drug concentrations after administration of the same dosage [1]. The genetic makeup could be the reason of variation in drug response among individuals [2]. In general, genetic factors are estimated to account for 15–30% of interindividual differences in drug response, but for certain drugs, this can be as high as 95% [1].

The genetic variations contribute to absorption, distribution, metabolism, excretion, response, and adverse drug reactions, which could be explored by pharmacogenetics (PG). PG could also characterize differential enzyme activity (e.g., the cytochrome P450 system) informing appropriate drug dosage on the individual (personalized medicine) and population levels. The advancement of genetic modalities will enable more accurate predictions of drug-related death determinations and contribute to the growing not only in forensic toxicology context but also in clinical settings [2].

The main application of molecular autopsy involves investigation of drug-related deaths by exploiting several molecular techniques, especially those of genetic nature [3].

The PG use as an adjunct for molecular autopsy would add to the understanding of potential genetic contribution to metabolism of certain drug (such as tramadol), thus enabling and improving the practice of *antemortem* drug therapy [4].

Tramadol (TR) is a worldwide used pain killer drug. Since TR was marketed, it has been widely prescribed because of its relatively safe profile among opioids. Nevertheless, intoxication can occur and overdose can lead to fatal outcomes. Surprisingly, in some fatalities for which death is attributable to TR alone, *postmortem* blood concentration levels overlap with the therapeutic concentration range (0.1–0.8 mg/L) [5–8]. These fatal cases might be explained by pharmacokinetic and pharmacodynamic properties of TR that are known to be influenced mainly by the CYP2D6 phenotype.

1.1 Tramadol (TR)

TR is a synthetic centrally acting analgesic drug worldwide used for the treatment of moderate to severe pain [9, 10]. TR is used as a pain killer in different types of pain such as osteoarthritic, endodontic or dental, chronic cancer, acute renal, neuropathic, and postoperative pain. It is also used in case of acute myocardial infarction, postoperative shivering in lower abdominal surgery, Brugada syndrome, and morphine allergy [11].

However, several precautions should be taken before TR prescription. It should not be administrated below the age of 16 years, and some forms as Ultram should be administrated above the age of 18 years. TR should not be prescribed during pregnancy, in case of lactating and expecting mothers, person with epilepsy, mental illness or suicide attempt, heart or respiratory problems, stomach or intestinal blockage, liver, kidney, or metabolic disease, TR sensitivity, addiction to drug or alcohol, intake of some drugs as sedatives, tranquilizers, narcotics, 2 weeks intake of monoamine oxidase inhibitor (MAOI), methylene blue injection, antibiotics, antifungal, and anti-HIV medications [11].

1.2 Epidemiological data

The changes in opioid consumption have been described worldwide. In Europe, they were characterized by an increasing use of TR. Between 2006 and 2015 in France, TR (alone or in combination use) were the second most commonly used mild opioids. There was an increased consumption of TR over the 10-year period (+62%) in France. In recent reports, France ranked third place for mild opioid consumption, with TR (alone or in combination use) being one of the most used substance (48%). In other European countries, TR is also the most commonly used mild opioids in Germany, Italy, Spain, and Denmark (98, 82, 78, and 85%, respectively) [12].

In the same time, there is a particular concern about the rise of nonmedical use of analgesics, especially opioids. Much has been written about the opioid crisis in the USA, but a similar crisis engulfing the Middle East, North, and West Africa is receiving little attention [13]. Therefore, drug world report 2018 mentioned the critical challenge in some countries in Africa and other regions needing to grips with the TR crisis [14, 15].

The 2017 report of the National Survey of Substance Abuse in Egypt was subsequently presented to the WHO which revealed that the abuse of TR is still a national concern in Egypt despite it became a schedule IV controlled substance since 2012. This may be because of wide-scale abuse of this analgesic. In the 2012/2013 survey, 7.6% of the general population abused drugs: of these 31.5% reported misuse of TR [16].

Because TR is widely used either as licit or illicit drug, FDA has only approved the medical use by prescription. Although TR has become a schedule IV controlled substance in the USA since 2014, this did not hinder legitimate access. In 2014, there

was a total of 43.7 million, 39.8 million, and 36.5 million TR prescriptions dispensed in the USA in 2016, 2017, and 2018, respectively [17].

1.3 Adverse reactions

The WHO Global Database reports of suspected adverse drug were studied to investigate TR. There has been a sudden increase in reports (nearly 5-fold) for TR: from 200 reports in 2013 to 800 in 2018 [9].

In overdose, the multiple systematic symptoms are reflecting the multimodal activity of TR. Acute adverse effects associated with TR are like those of other weak opioids. Common side effects include dizziness, nausea, constipation, and headache. TR overdose presented with multiple systematic symptoms ranging from cardiovascular toxicity to significant neurologic toxicity including lethargy, nausea, tachycardia, agitation, seizures, coma, hypertension, and respiratory depression [9].

The smallest amount of TR associated with a seizure was 200 mg occurred within 6 hours after ingestion. The mechanism underlying TR toxicity has been closely related to both opioid and MAOI activity [18]. The enhanced risk of seizure was attributed to the increased risk from serotoninergic toxicity due to the expected prolonged half-life of the ingested parent compound resulting in slower drug metabolism [19]. Both TR and M1 inhibit the reuptake of serotonin and noradrenaline. Hence, the concomitant use of serotonergic drugs such as serotonin reuptake inhibitors and MAOIs, increase the risk of adverse events, including seizure and serotonin syndrome.

The parent drug of TR causes sedation (but does not impair ventilation) and the M1 metabolite causes both sedation and respiratory depression [now termed opioid-induced ventilatory impairment (OIVI)] which is responsive to naloxone [20]. Both monoaminergic and opioid mechanisms contribute to this effect. In a case series study of TR overdose, respiratory depression occurs only in severe cases of overdose with very high doses [9, 21].

On the other hand, other work suggested that poor metabolizers (PMs) of TR tend to experience more adverse effects of the drug. The results showed that intermediate metabolizers (IMs) were found to have a statistically higher incidence of adverse drug reactions (dizziness, headache, nausea, sweating, and dry mouth) when compared with the groups that metabolize TR faster [ultra-rapid metabolizers (UMs) and extensive metabolizers (EMs)]. Other studies found no difference in term of adverse events such as nausea and vomiting between patients with the CYP2D6 UMs, PMs, IMs, and EMs [22].

Regarding the chronicity, the main problem observed was the significant increase in comorbid anxiety, depressive, and obsessive-compulsive symptoms while there was no increase in psychotic symptoms [9].

Concerning dependence potential, the International Narcotics Control Board reported in 2018 widespread misconceptions regarding TR among the general population in North Africa and the Middle East. Some consider TR to be a mood enhancer that increases sexual stamina and/or boosts energy during work. However, mood elevation is often reported and leads to the consumption of higher doses of the drug, psychological or physical dependence, and increased risk of overdose [9, 23].

The development of physical dependence to TR is dose-related, and administrations of supra-therapeutic doses lead to a similar dependence profile to morphine, whereas the risk of physical dependence is lower than prototypic opioids when low-dose TR is used over an extended period. However, these are not exclusively related to its opioid effects and may reflect withdrawal from catecholamine and serotonin receptors and present as atypical sequelae [9].

1.4 TR-related fatalities

In a report about a young Caucasian female admitted to hospital with refractory cardiac arrest and high levels of both TR and M1, the genetic analysis revealed the patient had a duplicated wild-type allele, indicative of a CYP2D6 UM phenotype. The event was specifically ascribed to the inhibition of noradrenaline reuptake and excessive blood adrenaline levels following binge-type ingestion of TR (to gain a "high") that led to strong myocardial stunning [24].

In France, the number of deaths related to TR toxicity increased from 32 in 2013 to 49 in 2017. TR was the first most commonly cause of death due to analgesics [25]. In Egypt, about 18% of fatalities are related to TR in the national poison center. TR was the second most commonly cause of death among cases attended in the poison center in 2012 [26].

2. Pharmacology

TR is a complex drug that is administered as a racemate with the (+)- and (−)-enantiomers of the parent compound and related metabolites showing various pharmacological effects. It is metabolized by polymorphic enzymes including CYP2D6 and CYP3A4 to its more potent metabolites particularly *O*-desmethyltramadol (ODT, M1) as well as *N,O*-didesmethyltramadol (NODT, M5) [9, 10].

2.1 Pharmacokinetics

TR is marketed as the hydrochloride salt. It is available in a variety of pharmaceutical formulations for oral, sublingual, intranasal, rectal, intravenous, subcutaneous, and intramuscular administration. It is also available in combination with acetaminophen, immediate-release and extended-release formulations. Tablets and capsules are the most commonly used and easily available formulations. The recommended daily dose is in the range of 100–400 mg. The maximum dose should not exceed 400 mg/day [9].

After oral administration, TR is rapidly absorbed (with a time lag of 30 min for capsules). The bioavailability of TR is around 70% after single-dose administration, but increases to 90–100% after repeated administration as a result of the saturation of the hepatic first pass effect [9, 27]. TR sustained release capsules had identical bioavailability to TR immediate-release capsules with lower peak concentrations and less fluctuation in plasma concentrations [9].

The analgesic potency of TR itself is about 10% that of morphine following parenteral administration but more potent if administered orally because of the activity of M1. The production of analgesia is consistent with M1 formation, which commences an hour postadministration and peaks 2–3 h later [9].

The TR volume of distribution has been reported to be 2.6 and 2.9 L/kg in male and female subjects, respectively, following a 100 mg intravenous dose. The plasma binding of TR is approximately 20% [28]. TR crosses the blood-placental barrier and a very small amount of the drug is excreted in breast milk [29]. TR is mainly excreted through the kidneys, the remaining being excreted in feces [30]. About 60% of TR dose is excreted as metabolites, meanwhile 30% is excreted in the urine as unchanged drug.

The elimination half-lives range of racemic TR and M1 have been reported to be about 5–7 h [31]. The longer elimination time of TR in case of overdose (about 9.24 h) gives an indication about the capacity limited of TR metabolism, which is dependent upon the rate of metabolism by the P450 enzymatic system [9].

2.2 Pharmacodynamics

TR acts in a multimodal fashion to bring about analgesia that involves the μ-opioid receptor system, the noradrenergic system, and the serotonergic system. TR has some affinity for the μ-opioid receptor, whereas the active hepatic metabolite, M1 has high relative greater affinity for the μ-opioid receptor [32]. The affinity of morphine for this same receptor is approximately 10–100 times greater than M1 and 300 times greater than TR. TR is approximately 10-fold less potent than codeine. A weak agonistic TR effect was revealed at the δ-opioid receptors, and a weaker TR affinity was shown at κ-opioid receptors. TR acts by other mechanisms on the central nervous system including monoaminergic activity through weak noradrenaline and serotonin reuptake inhibition to prevent pain transmission [33].

TR is administered as a racemate, with the (+)- and (−)-enantiomers of the parent compound and their respective metabolites displaying different effects to achieve synergistic pain relief. The (+)-enantiomer of TR is most potent in serotonin reuptake inhibition, while (−)-enantiomer is a noradrenaline reuptake inhibitor. The (+)-enantiomer of the M1 has the highest affinity and potency up on the μ-opioid receptors. It also exerts most of the opioid effects. The (+)-ODT is the most potent stereoisomer in relieving pain as well as in causing adverse effects [10].

TR is mainly metabolized by two pathways: *N*- and *O*-demethylation (phase I reactions) and conjugation (phase-II reactions). There are at least 11 known metabolites of TR (M1–M5). The metabolites *N*-desmethyltramadol (M2), M3 and M4 of TR have negligible affinity for the human μ-opioid receptor. The *O*-demethylation of TR to M1 is catalyzed by cytochrome P450 (CYP) 2D6, whereas *N*-demethylation to M2 is catalyzed by CYP2B6 and CYP3A4 (**Figure 1**) [9]. M1, the active metabolite of TR, is metabolized through glucuronidation in the liver, mostly via UGT2B7 and UDP glucuronosyltransferase 1–8 (UGT1A8) [22].

2.3 Pharmacogenetics (PG)

2.3.1 Definitions

PG is the inherited variation study in relation to drug response. Its goal is to develop novel ways to minimize toxicity and maximize drug efficacy for the patient [34]. PG is an important innovation in clinical medicine as a result of progression of genomic science to determine the correct drug with the correct dose as a specific treatment with the use of genetic information [35]. Personalized medicine has the potential to select the most appropriate drug for certain patients, to predict optimal dosage for a drug, and to develop cost-effective treatments. Individualized response to treatment may be attributed to biological (for example age, sex, nature of disease), behavioral (for example smoking, drug interactions), or genetic factors (for example genetic variants of pharmacogenes) [34].

2.3.2 PG strengths

Personalized medicine has the finality of improving drug safety and efficacy. PG application in clinical practice promises more effective decision-making in relation to diagnostic testing, individualized drug selection, and dosing. This depends on the genetic variations that affect the observed differences in drug response which can be classified into two groups: pharmacokinetic and pharmacodynamic. The genes that influence the pharmacokinetic properties of a drug influence the drug absorption, distribution, metabolism, and excretion. The genes that affect the pharmacodynamic of a drug influence the mechanism of the drug's target and how

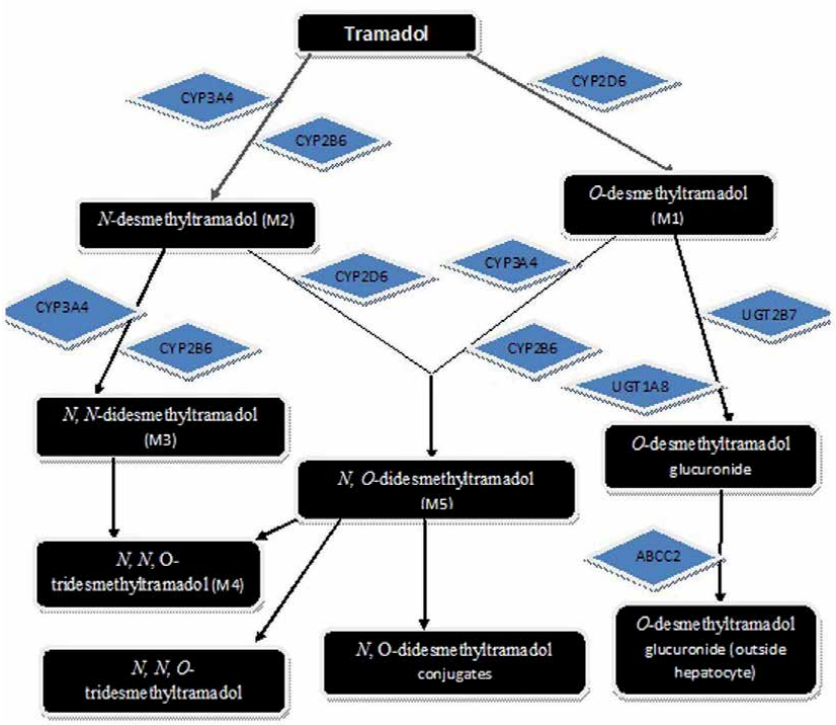

Figure 1.
Tramadol metabolism.

it affects the body. The risk-benefit balance of a drug can be evaluated based on PG effects on safety, efficacy, or both. Thus, prior analysis of a patient's genotype may be used to guide clinical decision because the patient may benefit from an alternative drug or reduction in dose of a standard therapy. PG has the potential to reduce the costs (pharmacoeconomy) associated with inappropriate drug or severe adverse drug reactions that require hospitalization [34].

2.3.3 PG limitations

There are several limitations of PG that invariably makes genetic-guided therapies unfavorable in comparison with the standard health care. One of these limitations is the cost of PG test as the additional expense of the genetic test has to be borne as an extra expenditure by the patient, or by healthcare coverage provided by insurance. The second common limitation is the speed of getting genetic test result as the speedy generation of the PG test result is as important as of the genetic information results itself. Imperfect understanding of genetic determinants of drug response is one of the common limitations which in turn affects physician and patient confidence to genetic test [35].

2.3.4 Opportunities for growth of PG

The landscape of PG testing is rapidly evolving. There are several factors that have great impacts on PG development and increase its utility such as accumulations of PG data, the continuity of technological innovations with more accessibility, and costs continuing to drop [35, 36]. Work has been done to suggest that genetic variations may be studied in oral fluid [33]. Oral fluid tests may encourage

healthcare professionals to use pharmacogenetic tools more often. Moreover, one of the PG-guided clinical trial principle is to improve and accelerate drug development by correlating genetic patient profile with treatment outcomes in early clinical trial phase, and subsequently extending Phase III only to individuals having the genetic predispositions linked to the safe and effective use of the tested drugs [35].

2.3.5 Uses of PG

PG can enhance patient care by applying treatments tailored to genetic make-up and decreasing the risk of severe adverse effects. In 2019, there are 132 PG dosing guidelines for 99 drugs and PG information is included in 309 drug labels. Recently, the genotyping has become more accessible. Next generation sequencing is a cost-effective choice to genotype samples at many PG loci simultaneously [37], and guidelines are available from organizations such as Clinical Pharmacogenetics Implementation Consortium and Dutch Pharmacogenetics Working Group [36].

As mentioned earlier, the major P450 enzyme activities involved in drug metabolism are influenced by genetic variations. Thus, it is not surprising that the currently available examples of the beneficial use of PG in the interpretation of forensic toxicology data predominantly concern drugs that are extensively metabolized by P450s. Many cases exhibited severe intoxication or even death can be attributed to genetic variations in drug-metabolizing enzymes leading to toxic concentrations of either the parent drug or metabolite(s) in the body. Therapeutic opioids (as codeine, TR, oxycodone, hydrocodone, ethylmorphine, and methadone), selective serotonin reuptake inhibitors (as fluoxetine, fluvoxamine, paroxetine, sertraline, and citalopram), and tricyclic antidepressants are commonly implicated in severe adverse effects, as well as drug-related deaths because of certain genetic polymorphisms which affect the drug metabolism [38].

2.3.6 PG and TR

The CYP2D6 enzyme is accountable for the formation of M1 (**Figure 1**). Individuals may be classified as PM, IM, EM, or UM according to the metabolic activity of the CYP2D6 enzyme, determined by either phenotyping or predicted from genotyping (**Table 1**) [10]. CYP2D6 genotype data are commonly arranged into star (*) alleles. The intermediate (extensive/normal commonly divided into fast [NM-F] and slow [NM-S] subgroups) [39]. Individuals with two functional CYP2D6 alleles or one functional and one decreased functional allele were classified as EM. Individuals with one nonfunctional allele and one functional or decreased functional allele are identified as IM [10]. A carrier of two reduced function is also considered IM [22]. Individuals with two nonfunctional alleles as PMs. UMs were defined as individuals with CYP2D6 multiplications, resulting in at least three functional CYP2D6 alleles [10].

Different pharmacological effects can therefore be expected depending on an individual's CYP2D6 phenotype. Both pharmacological properties (opioidergic and monoaminergic) of the drug may also be modified because of modulation of P450 enzymatic activity by genetic polymorphisms and/or drug interactions. The opioidergic properties will be very clear in UM, meanwhile the monoaminergic antidepressant activity will be very clear in PM [40].

The PM, in comparison to EM, have an increased exposure to (+)- and (−)-TR combined with a reduced formation of especially (+)-ODT but also of (−)-ODT. UM, on the contrary, are expected to form higher amounts of (+)-ODT than EM and to be more prone to opioid- related adverse effects. Comparisons between EM and UM are, however, sparse in scientific literature [10]. PMs are experiencing better pain relief

Phenotype	Genotype (allele)		
	Fully functioning	Defective functioning (decreased, reduced)	Nonfunctioning (dysfunction-inactive)
UM	≥3	0	0
EM			
EM1s (heterozygous wild-type)	1	1	0
EM2s (homozygous wild-type)	2	0	0
IM	1	2/1	1/ 1
PM	0	0	2
Examples	*1	*9, *10, *17(Z)	*3, *4, *5, *6, *14

UM, ultra-rapid metabolizer; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer.

Table 1.
CYP2D6 phenotypes in relation to their genotypes.

than IMs who may experience insufficient relief. UMs are being more likely to experience adverse effects from TR because of the more rapid release of M1 [9].

Apart from M1, there are two additional primary TR metabolites, M2 and M5. M2 is an inactive metabolite which its formation is mediated by presence of two enzymes (CYP2B6 and CYP3A4), while M5 has some opioid effects and its metabolic route is less assured. However, all three enzymes (CYP2D6, CYP2B6, and CYP3A4) are involved in TR metabolism (**Figure 1**) [10].

The CYP2B6 gene is highly polymorphic as well as CYP2D6, but the relevance of CYP2B6 polymorphisms in TR has been less studied. Many CYP3A4 polymorphisms have been identified, although it is not well associated with the phenotypical variability. The CYP3A4*22 allele is associated with reduction of the enzymatic activity. Recent study tried to explain if the interindividual differences in enantioselective metabolic profiles could be explained by CYP2D6, CYP2B6, and/or CYP3A4 genotype. This item hypothesized that interindividual differences are better explained by the combined genotype of all three enzymes involved in the metabolism of the drug, rather than by CYP2D6 itself [10].

Rudaz et al. showed that enantiomer ratios of all four compounds in urine changed over time in one individual administered 100 mg TR [41]. The largest increase in enantiomer ratio was observed for M2 in CYP2D6 EM and IM, rising from about two to almost seven during 24 h following drug intake. Homozygosity of CYP2B6*5 and *6 indicated a reduced enzyme function, although further studies are required to confirm it. The significance of CYP2B6 polymorphisms in TR pharmacokinetics has not been carefully investigated. The same study concluded that consideration for the time is important when you assess enantiomer ratios as it might possibly be used to distinguish a recent TR intake from a past one [10].

Of additional relevance is the significant interethnic differences in CYP2D6 allele frequencies demonstrated across many countries [9]. For example, the CYP2D6*1 seems to be the most prevalent in studied groups from Egyptians. In contrast, CYP2D6*4, CYP2D6*10 and CYP2D6*DUP showed minor occurrence [42].

Chronic treatment with TR induced hepatotoxicity in all patients with duplicated or normal function (CYP2D6*DUP or *1) allele in UMs and EMs displayed higher blood levels of M1, but in none of the patients with impaired or reduced functioning allele *4 or *10 [42].

3. Detection of TR

3.1 Techniques

Toxicological analysis of TR is based on chemical spot tests, immunoassays, mass spectrometry, and chromatography.

- Preliminary urine multidrug screen tests as well as automated immunoassay systems which are commonly used with a 0.3 mg/L cut-off value [43].

- Gas chromatography-mass spectrometry (GC-MS) can be used to analyze blood samples for detection of TR and its metabolites. Blood concentrations are ranging from 0.03 to 22.59 mg/L, from 0.02 to 1.84 mg/L and from 0.01 to 2.08 mg/L for TR, M1, and M2, respectively [9, 44].

- Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantitation of TR and its main metabolites in hair. The Lower detected Limits were in the range 0.010–0.030 ng/mg hair. The TR, M1, and M2 concentrations were markedly lower in the nonabuse cases (3.3–20.1 ng/mg, 0.3–1.9 ng/mg, 0.5–4.3 ng/mg, respectively) compared to the abuse cases (63.4–107 ng/mg, 3.8–6.3 ng/mg, 24.9–45.7 ng/mg hair, respectively); also the M2/M1 ratio differed significantly. Hair has become an important biomarker owing to the possibility of detecting target analytes for periods >1 month [45].

- Liquid chromatography coupled to fluorescence detection was also used for TR and its main metabolites M1 and M2 and M5 simultaneous determinations in human plasma, oral fluid, and urine. The lower limit of detection was 2.5 ng/mL for all compounds. The assay was applied to assess the pharmacokinetics of TR and its main metabolites following administration of a single oral dose (100 mg) TR to healthy volunteers [46]. Maltodextrin-modified capillary electrophoresis method was reported to detect the stereoisomers of TR for a single-run chiral separation of TR with detection limit of 2 mg/L. This method was approved to measure the concentration of drug in plasma samples, urine, and tablets [47].

- HPLC (linear dual column-MS/MS) was successfully used on oral fluid for the simultaneous detection of TR and its metabolite (M1, M2, and M5) [33].

3.2 Interpretations of toxicological and genetic analyses

Different studies tried to correlate concentrations of TR and its metabolites with different genotyping of CYP2D6. The best correlation was obtained for M2/M1 ratio and PM. The M2/M1 ratio in PM is almost above 7 [48].

Levo et al. calculated the TR/M1 and TR/M2. When the number of functional alleles increased, the median TR/M1 decreased. They also showed that median TR/M2 also correlated with the number of functional alleles, but in the reverse direction, as can be expected based on the complementary nature of two pathways [49].

The literature reported the value of M1/M2 ratio in case of massive TR intoxication. A quick death has been expected in case of M1/M2 is more than 1 [50].

The metabolic profiles of CYP2D6 PM showed large area under curve (AUCs) of the M2 enantiomers with low corresponding values of the M1 and M5 enantiomers. The (+)-enantiomers of M1 and M5 were affected to a larger extent than the

(−)-enantiomers. The observed reduced levels of the M5 enantiomers were expected. M5 is formed from both M1 and M2. Since PMs only form low amounts of M1 due to the abolished (canceled) function of CYP2D6, the amounts of the M2 enantiomers will accumulate. Another metabolic profile of CYP2D6 IM showed the AUCs of both the M2 and M5 enantiomers exceeded the ones of the M1 enantiomers [10].

The general hypothesis in literature regarding adverse effects following TR administration is that the frequency and intensity is related to the concentrations of (+)-M1. The higher the concentration of (+)-M1, the higher the risk of side effects and toxicity. In Haage et al. study, the individual experiencing most drug reaction symptoms (both fainting and vomiting during the experimental day) was the one with the second lowest maximal concentration and AUC of (+)-M1 in the 100 mg dosage group [10].

A recent work reported that the TR/M1 ratio may not accurately reflect the rate of TR *O*-demethylation in clinical patients. This study accounted that due to (i) *postmortem* redistribution (PMR) phenomenon, and (ii) various interval and/or time between TR administration and death. In addition, TR is metabolized by multiple enzymes (e.g., TR and/or its metabolites are metabolized by CYP2D6, CYP3A4, CYP2B6, UGT1A8, or ABCC2) (**Figure 1**) and may be metabolized at different rates based on body size, liver function, chronicity of TR, general opioid, and/or other drug use [39]. Surprisingly, the first abovementioned cause is not accurate as PMR is not a problem in case of interpretation of fatalities related to TR. Indeed, many studies revealed that cardiac-to-femoral blood ratios obtained for TR and M1 are close to 1 [51–54].

It is suggested that the monogenic model likely introduces error, particularly for samples at the extremes of CYP2D6 activity; a prediction using multiple genes may reduce these discrepancies although this comparison has not been performed. The forensic community has not yet leveraged the power of machine learning for such studies. However, the forensic DNA community has begun developing methods of individualizing humans [39].

4. Conclusion

This chapter about PG and TR highlights important related issues including pharmacological and genetic aspects of TR. It clarified the interindividual variability in response and toxicity to TR in order to support the use of genetic screening to predict individual responses to pain medications and the risk of adverse events. This in turn will encourage the use of PG as part of clinical practice for TR. Finally, novel machine learning approaches using multigenic model can comprehensively analyze genotype-phenotype relationships that hold significant promise for application to predict response to TR, which may contribute to the relatively high rate of TR-related fatalities.

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Conflict of interest

The authors declare no conflict of interest.

Appendices and nomenclature

EMs or NMs	Extensive or normal metabolizers
GC-MS	Gas chromatography-mass spectrometry
IMs	Intermediate metabolizers
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
M1 or ODT	*O*-desmethyltramadol
M2	*N*-desmethyltramadol
M5 or NODT	*N,O*-didesmethyltramadol
MAOI	Monoamino oxidase inhibitors
NM-F	Fast normal metabolizers
NM-S	Slow normal metabolizers
OIVI	Opioid-induced ventilatory impairment
PG	Pharmacogenetics
PMs	Poor metabolizers
TR	Tramadol
UMs	Ultra-rapid metabolizers

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Chapter 11

Herbal Drugs Forensic

Shalvi Agrawal and Astha Pandey

Abstract

Due to pandemic Covid-19, suddenly the vast population is drawn towards herbal drug treatment in India. In India, Ayurveda is practised to a greater extent as it does not have any side effects or other major effects. They are also added in many nutraceutical products like Chyawanprash, honey, etc. There are a lot of medicinal floras sold in the market in the form of small twigs, pieces of roots, stems or leaves of which decoction is made and consumed. The consumers are unaware of the authenticity of these crude drugs that lead to the deterioration in their health owing to the consumption of inferior quality of products or their substitute or the illicit bioadulterants which look like any other common plant part. The herbal drugs could also be in the form of tablet, powder, etc. which might be adulterated with look-alike plant products. Thus, a new branch of **Forensic Science,** i.e. **Herbal Drugs Forensic** which deals with identification of fake herbal product by various techniques which might be chemical or biological in nature has come up. In fact, the analytical methods for the testing of various bioconstituents need to be standardized and validated. Thus to prevent herbal drug fraud, it has become necessary to develop the methods for their detection through an emerging field of Forensic Science, i.e. Herbal Drugs Forensic.

Keywords: adulteration, authenticity, fraud, herbal drugs, herbal drugs forensic, identification, standardization

1. Introduction: Ayurveda - the science of healing

> *'The great thing about Ayurveda is that its treatments always yield side benefits, not side effects.'—Shubhra Krishan*

Ayurveda is an age old science that deals with the sacred knowledge of healing and longevity. The word Ayurveda is derived from two words that is 'Ayuh' which means 'life' and 'veda' which means 'science or knowledge'. Thus, Ayurveda itself defines its meaning as the science or knowledge of life. Not only a science, but Ayurveda is a tradition that originated in India and is practised since then.

In other words, Ayurveda is not only taking herbal preparation and waiting for it to give results but encouraging all of us to be an active participant in the journey of healing. This process involves learning about our relationship with the nature and elements and their unique combinations they form known as *doshas*.

The practice of Ayurveda dates back to over five thousand years, during the *Vedic* period of ancient India [1]. The tradition was kept alive by the *vaidyas* and *acharyas* (those who practise Ayurveda) till India got independence in 1947. After independence, Ayurveda saw a resurge in India and emerged as a major healthcare system. Not only in India, but in different parts of the world like China, Europe,

Middle East, etc., Ayurveda has been practised since long as the alternative medicine system was not in reach of major population [2].

Along with Ayurveda, practice of Yoga and eastern spiritualism flourished towards the western world in twentieth century.

1.1 The positive side of Ayurvedic medicine

The most important advantage associated with ayurvedic medicines is that it gives no side- effects even if it does not cause any good to health. This property of Ayurvedic medicines makes it most popular among all systems of medicine. The practice of Ayurveda is as easy for the new practitioners as it is for the age old [3]. It does not work on the symptoms of the disease rather works on the root cause to treat the ailment permanently. That is why the practice of Ayurveda requires patience.

1.2 Ayurveda and the current pandemic

Antibiotics resistance across the world is already a problem, especially if Covid-19 deepens as a syndemic in populations with antibiotic resistance [4, 5]. To fight with such a situation, Indian Ayurvedic system of healing can play a vital role. Also, against such a syndemic, there should be something that can act synergistically. For such a synergy, a combination of drugs can prove in a most effective manner that can not only help in fighting against such a syndemic but can also play an efficient role in boosting the immunity and acting as a line of defense against any such future epidemic.

2. Forensic botany and herbal drugs forensics

The study of plants and their role in criminal investigations is referred as 'Forensic botany' [6, 7].

Forensic Botany not only involves the examination of botanical evidences present near the victim or at crime scene but also involves the detection of any unwanted material present in the herbal drugs. Though this part is untouched in forensic botany and is considered differently under Ayurveda but whenever and wherever any illegal, intentional or unintentional substitution or adulteration in trade of natural/authentic product comes, forensic science has always a crucial role to play.

A fundamental Ayurvedic philosophy is that 'food is medicine and medicine is food'. According to an Ayurvedic proverb 'When diet is wrong, medicine is of no use; when diet is correct, medicine is of no need'.

Food, the fundamental for the sustenance of life is not unblemished from various malfeasances owing to the adulteration and falsification of food commodity. Not only food but the drugs also are no safer enough to be completely relied upon. The food and the herbal drugs (prepared from the medicinal flora having one or more bioactive constituents) market offer a huge income due to growing population, their needs, consumerism and marketism. The various food products and herbal drugs available in the market are carrying lot of adulterants and counterfeits that are almost impossible for a common man to detect [8–14]. **Herbal drugs forensic** is a branch of forensics and a multidisciplinary science that not only answers the questions as to 'what' and 'how much' related to drug safety and quality issues but also investigates the sources, fate, implications and possibilities related to adulteration, falsification, counterfeiting and substitution in nutraceuticals (the drugs or herbal supplements consumed for extra nutrient intake that is not fulfilled by routine diet) and herbal medicinal drugs.

3. Trade in herbal drugs

According to National Medicinal Plant Board (NMPB) Report, India's domestic herbal industry (since India being a major exporter of herbal raw drugs) has around 8610 licensed herbal units, thousands of cottage level unregulated herbal units and millions of folk healers and household level users of thousands of herbal raw drugs. Also, the trade web that channelizes various herbal raw drugs from the supply to the end users is very complex. Thus, to have a better understanding of the Market and trade of the sector, we need to focus on demand and supply chains of the medicinal plants. The demand and supply chain of medicinal plants in the country is itself very complex.

Presently, medicinal plants are marketed and traded through Mandis and other wholesale markets in India with numerous intermediaries in between. Trade is rather opaque and information on prices, arrivals and other trends are difficult for farmers/growers to access. NMPB has been initiating many steps in order to fill this gap.

Another major gap between the demand and supply has emerged due to the commercialization of the production of classical Ayurveda, Siddha and Unani (ASU) formulations that require large quantities of wild harvested, cultivated or imported herbal raw drugs. This has resulted into thriving trade in raw drugs. Because of this, knowledge about the annual consumption levels of the herbal raw drug, the trends in their usage and their trade value is important so that the resources can be managed to ensure sustainable supplies to the herbal drug industry, folk users and growing global markets [15].

3.1 Demand and supply position of medicinal plants

India has very strong traditional health care practices that are represented by the classical systems of medicine like Ayurveda, Siddha, Unani, and Swa-rigpa. Besides these traditional heath care practices, there exists a very diverse area-specific and community-specific folk healthcare practices. Both the Indian classical system of medicine and the folk health care systems are highly dependent on the raw herbal drug material derived from a diverse species of medicinal plants, which is estimated to be about 6500 in number [16].

NMPB, during 2001–2002, commissioned a study through Centre for Research, Planning and Action (CERPA) to understand annual trade levels of selected 162 medicinal plant species. The NMPB, then in 2006–2007 commissioned a national study that was carried out by Foundation for Revitalisation of Local Health Traditions (FRLHT) to assess demand and supply of medicinal plants in India which for the first time highlighted various drawbacks in the herbal drug sector and added more to the existing knowledge and understanding of the subject related to the diversity of raw drugs in trade, their botanical correlation, volume of annually trade drugs, their supply sources and others [16].

Figure 1 below shows various Industrial uses of Medicinal Plants [17]. Each of these groups can contain a wide range of products like herbal medicinal products, food supplements or dietary supplements, foodstuffs, Cosmetics, etc.

Countries where the highest uses of traditional medicines are practiced include [18]:

- United Arab Emirates (100%)
- China (100%)
- India (70%)

- Pakistan (70%)
- African countries (70–80%)

A major percentage of raw drugs are used in making plant extracts. This is carried out either by the end product manufacturers or by extract companies. In addition to this, there is an exorbitant demand in developed countries for plant based products including health supplements, herbal health drinks, herbal cosmetics and various other health and personal care products. The current worth of the global market for herbal products is estimated around US$60 billion per year with a growth rate of 7% [16].

In India, trade in medicinal plants accounts for a turnover of Rs. 2300 crores of ayurvedic and herbal products with a contribution of about Rs. 1200 crores of over-the-counter products. Currently Covid-19 pandemic has opened door to explore more in the ayurvedic treasures. This would help in enabling rapid access of these herbal drugs into developed country markets as well as in Indian market [16].

There are major challenges in tapping the substantial potential for utilizing medicinal, aromatic and natural dyes plants (MADPs) nationally in India, as well as in export markets. At the forefront of these problems is ensuring consistent and acceptable quality which always suffers against making money out of it. As this tradition has now moved to industries which was earlier a part of a local community's culture and health practices, quality is not manageable and has suffered a

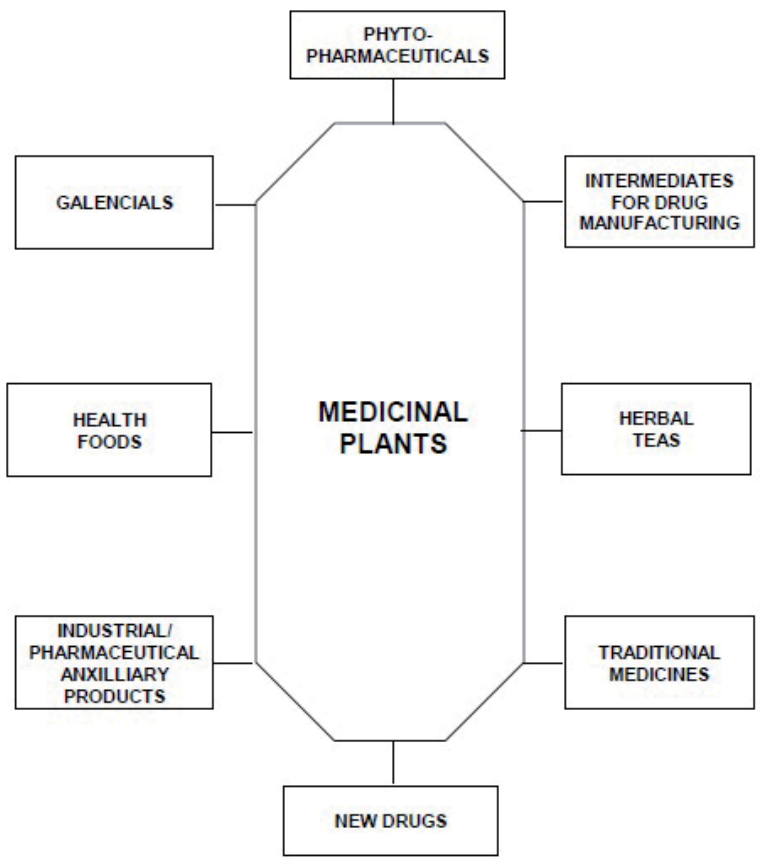

Figure 1.
Industrial uses of medicinal plants. Source: De Silva [17].

lot. In case of medicinal plants, quality means the correct identity of the medicinal herb, purity of the composition and safety and efficacy of the final drug. However, quality standardization of MADPs suffers a lot of challenges as:

- Firstly, unlike allopathic drugs that are composed of a single or a fixed number of chemical compounds that are easy to be standardized, plants have a diverse range of phytoconstituents contributing to their bioactivity and it is very difficult to isolate, purify and standardize that bioactive compound.

- Secondly, bioactivity is not constant but varies according to time (day, season, constellar position) and to region (arid, marshy).

- Thirdly, there are also differences in the bioactivity exists depending on the way a plant has been collected, processed and stored [16].

There are many more factors that contribute to the varied behavior of plant based drugs besides the ones stated above. Phytoconstituents that are responsible for the specific medicinal properties of a plant are not present in uniform quality or quantity under different edaphic (soil) conditions. The medicinal and toxic property of a plant may also vary according to the kind of processing through which it had undergone. Traditional, ancient systems of medicine used to take care of all these parameters and thus it had specific recommendations and procedures as to how a plant should be collected and processed in order to get its optimum efficacy.

Currently, there are modern analytical techniques like chromatography, spectroscopy and bioassay methods that can be used to determine the chemical profile of the selected medicinal plants and can compare their bioactivity and efficacy in treating one or the other disease [16].

Note: Information in the above paragraph has been taken from the country studies prepared for the Workshop on Medicinal Plants held in Bangalore, India, 22–26 July 2004.

4. Constraints to the development of trade

While developing countries like India has huge potential in the marketing and trade of herbal drugs owing to their ancient knowledge and experience with the Ayurveda but they face a number of challenges and limitations that need to be addressed to meet the growing demands in the developed country markets [16].

a. **Quality assurance:** the herbal drugs need to be consistent in quality and should be free from contaminants. Unwanted ingredients like dirt, soil particles, plant parts, or any other chemical adulterant need to be addressed before final packaging and trade of the herbal product.

b. **Consistent volume:** the herbal drugs that are high in demand must be available in consistent volume so that there should be no pressure on natural resources and no exploitation of biodiversity occurs.

c. **Strict regulatory laws:** to regulate the collection, processing, storage, manufacturing and trade in herbal products, there should be strict norms laid by the concerned authority at local, national and international level.

d. **Pre- and post-harvesting practises:** the herbal plants that are harvested for the use in different products need to be monitored and their pre- and

post-harvesting practices must be standardized so that a consistent quality can be met. Also, as we discussed earlier, quality and quantity of bioactive components are impacted depending upon pre- and post-harvesting practices.

e. **Lack of proper knowledge:** lack of proper knowledge in the species and variety of the plants many a times create confusion. There is limited knowledge of the herbs' medicinal properties beyond traditional knowledge and belief. This restricts the use and marketability of the plants. Common man/consumers are unaware of these scientific terms and blindly trust the manufacturer or brand in this regard.

f. **Lack of database/data repository:** there must be a herbal drug repository and related database of all the medicinal plants found in the country, their availability, flowering season, abundance, medicinal properties, toxicity, etc. so that exploitation of natural resources can be stopped and endangered species of plants can be protected.

g. **Lack of access to latest technology:** developing countries/Poor countries often lack in accessing latest technologies for the quality assessment of the herbal drugs which results in compromising with the quality and efficacy of the drugs.

h. **Research and development:** often developing countries use a very less proportion of their Gross Domestic Product (GDP) in research and development. In case of herbal drugs, research and development is the foremost requirement as the drugs are sourced from natural product and the environment in which these plants grow is ever changing with lot of mutations occurring resulting in change of bioactivity of phytoconstituents.

i. **Intellectual property rights (IPR):** plants cannot be protected under IPR as they have been used in traditional medicines for centuries. Any new discovery with a herbal drug that show its effectiveness in treating certain ailment can be patented. A therapy which is new for one region can be a part of regular practice for the other region of the world. Thus, everything cannot be patented. The plants can only be registered based on their availability in one or the other region and their status in the wild. Also, there is very little knowledge of the whole IPR field in the developing countries and the access to it is still lower. These issues are currently under discussion, debate and negotiation on a broader scale in the World Trade Organization (WTO).

j. **Deliberate malpractices:** beside lack of knowledge, there is sometimes deliberate adulteration and substitution in herbal drugs to make more money. Always the end users pay a cost of these malpractices and sometimes suffer due to inferior quality of the products which many a times produce side-effects.

A detailed review on the various malpractices in herbal drugs is discussed below.

4.1 Types of illegal/malpractices

Deliberate malpractices can be broadly classified into two categories- **adulteration** and **substitution**. The term '**adulteration**' can be defined as 'admixturing or substituting original or genuine drug with inferior, defective or otherwise useless or harmful substance'. Adulteration in simple words is the debasement of an article [19]. The drugs may be adulterated with substandard commercial varieties or with superficially similar inferior drugs or

with artificially manufactured substance or with same exhausted drug or with synthetic chemicals to enhance natural character or with any other harmful adulterant. The drugs which are in powdered form are frequently adulterated as identification of adulterant becomes very difficult in powdered form of drugs. According to World Health Organization's (WHO) publication on quality standards for medicinal plant materials, any batch of raw herbal drug which has more than 5% of any other plant part, even of the same plant say, stem in leaf drugs, should be strictly rejected never the less if they are derived from the authentic plant.

Substitution may be defined as a process of replacing partially or completely an authentic herbal drug with its different species or with different plant part or with any other material that have similar morphology or functional property so that the substitution may be difficult to detect.

4.1.1 Types of adulteration and substitution

a. *Adulteration with inferior commercial varieties* [20, 21]: they are added due to similarity in morphology; for example, black pepper (*Piper nigrum*) adulterated by papaya seeds.

b. *Adulteration by artificially manufactured substitutes* [20, 21]: the artificially manufactured substance resembles the original drug. This method is used for the costlier drugs. For example, artificial invert sugar or cane sugar is added to Honey.

c. *Adulteration by exhausted drugs* [20, 21]: when the drug is devoid of medicinally active substance as it has been extracted already and then is used to make the herbal product, such substitution comes under this category. Mainly volatile oil containing drugs like clove, coriander, fennel, caraway are adulterated by this method. As it is devoid of colour and taste due to extraction, natural colour and taste is balanced with additives and synthetic chemicals, e.g. Clove, Fennel, etc.

d. *Adulteration by addition of heavy metals* [20, 21]: many a times due to non-standardized or sub-standardized processing of raw herbal drugs, many harmful pollutants from the soil and environment get added to the final product, e.g. pieces of limestone in asafoetida, lead in pieces of opium.

e. *Adulteration by synthetic principles* [20, 21]: synthetic chemicals are used to enhance natural colour, fragrance and taste of the exhausted drugs, e.g. adding Citral to oil of lime and orange.

f. *Adulteration of powders* [20, 21]: the drugs which are in the form of powders are frequently adulterated as it is almost impossible to detect the adulteration without any technological tool. For example, dextrin is added in ipecacuanha, exhausted ginger in ginger, red sanders wood in capsicum powder and powdered bark adulterated with brick powder, metanil yellow in turmeric powder, chalk powder in wheat flour, red brick powder in red chili powder, chicory powder in coffee powder, etc.

g. *Presence of vegetative matter of same plant* [20, 21]: small pieces of all dried root, stem and leaf of different plant look similar. Some miniature plants growing along with the medicinal plants are added due to their colour, odour, and constituents. Small twigs, stems, leaves are often added to the

crude dried drug which cannot be differentiated by naked eye. For example, Mixture of crude Dashmoola drugs is often adulterated with some random twig, leaf and bark.

4.1.2 Reasons for adulteration and substitution

a. *Non-availability of the authentic drug* [20, 21]: due to over exploitation and unregulated deforestation, there has been shortage of many medicinal floras which has resulted in the endangerment of the species. Some of the plants have been extinct also, e.g. substitution of *Oroxylum indicum* in Dashmoola drugs.

b. *Uncertainty in the identity of the drug* [20, 21]: the local collectors of the medicinal flora are not much educated and get confused with the identity of the drugs that look similar. Also, some of the plant species are debatable among Ayurveda practitioners for the authenticity and thus interchangeably used by different practitioners, e.g. for the herb Lakshmana different species such as *Arlia quinquefolia*, *Ipomea sepiaria*, etc. are considered.

c. *Cost of the drug* [20, 21]: due to lesser prevalence of many medicinal floras, the cost of their respective drug rise. Manufacturers adulterate such drugs to make more money, e.g. saffron, asafoetida, etc.

d. *Geographical distribution of the drug* [20, 21]: many of the drugs that are found in one part of the country are not found in the other. This has resulted in substitution of such drugs with functionally similar drugs. For example, as Rasna, *Plucia lanceolata* is used in Northern India while in southeren parts *Alpinia galanga* is considered as the source.

e. *The adverse reaction of the drug* [20, 21]: different drugs have their varied impact on people depending upon their age, gender, physical condition, disorder, etc. Some of the drugs that show medicinal benefits in one person may show toxic effects in another. For example, Vasa is a well-known Rakta-Pittahara drug, but due to its Abortifacient activity its utility in pregnant women is limited, instead drugs such as Laksha, Ashoka, etc. are substituted.

f. *Confusion in vernacular names* [20, 21]: different species having similar vernacular names and vice a versa an cause confusion that may result in adulteration. In Ayurveda, 'Parpatta' is a plant that refers to *Fumaria parvifloran* originally and in Siddha, 'Parpadagam' is another plant that refers to *Mollugo pentaphylla*. But due to similarity in the names in traditional systems of medicine, these two herbs are often interchanged or adulterated or substituted [19].

g. *Lack of knowledge about original or authentic source* [20, 21]: an example of such type of adulteration can be *Mesua ferrea* commonly known as Nagakesar. Market samples of Nagkesar is adulterated with flowers of *Calophyllum inophyllum* because the suppliers are not aware about the abundance of authentic drug *Mesua ferrea* in the Western Ghats region and the parts of Himalayas. There may be some restriction s in the collection of drug from the protected forests too [19].

h. *Similarity in morphology* [20, 21]: owing to the similarity in morphological characteristics within the same genus, often the species are interchangeably used. *Mucuna pruriens* is adulterated with other similar Papilionaceae seeds

having similarity in morphology. *M. utilis* (sold as white variety) and *M. deeringiana* (sold as bigger variety) are popular adulterants.

i. *Similarity in colour or dye* [20, 21]: colour can be a major reason of adulteration. Not all spices and drugs that give a peculiar colour to food or medicine source from original herb but can be adulterated with synthetic dyes. 'Ratanjot', originally derived from *Ventilago madraspatana* and collected from Western Ghats which is known to be the only source of red dye (ratanjot), is now derived from *Arnebia euchroma* var. *euchroma*. Though there is only similarity in the colour of the dye but still, whatever is available in the market, in the name of Ratanjot is originated from *Arnebia euchroma* [19].

j. *Careless collections* [20, 21]: some of the herbal adulterations are due to the carelessness of herbal collectors and suppliers. The collectors are local people who are not much educated and trained in proper collection guidelines. This results in degradation of many of the useful properties of the plant. *Parmelia perlata* is used in Ayurveda, Unani and Siddha. It is also used as grocery. Market samples showed it to be admixed with other species (*P. perforata* and *P. cirrhata*).

5. Laws related to collection, harvest, processing and trade in medicinal Flora

As discussed earlier, with the growing interest in medicinal plants and ayurvedic drugs, there is a need for a long term strategy and planning to conserve and sustainable use of these plants [22–26]. Many medicinal plants like other natural resources are facing extinction and their degradation has accelerated over the years due to many reasons. Medicinal plants also face habitat destruction due to over exploitation. Though **Forest Conservation Act, 1980** and the **Wildlife Protection Act, 1972** provide some protection to medicinal plants. But as there are many medicinal floras that grow away from the domain of protected areas and there is no well-planned strategy for their conservation, many of such unchecked floras face endangerment and become extinct. Even the floras within protected areas face depletion without proper conservation strategy. Beside this, indiscriminate and unregulated exploitation of medicinal plants for their roots, stem, bark, fruit, leaves, or whole plant leads to destructive harvesting which include 70% of the wild flora out of 95%t used [16]. If not carefully monitored, this practice could lead to the depletion of genetic stocks and ultimately to the diversity of medicinal flora.

Though, government is making efforts to manage and regulate the collection of wild flora, but a long term strategy is needed. The efforts are scattered and do not yield satisfactory results. These efforts along with the well-planned strategies and policies are needed with major involvement of local communities and indigenous habitants.

Both in-situ and ex-situ conservation strategies need to be undertaken. **In-situ conservation** involves protecting bioreserves and biodiversity hotspots, e.g. The Himalayas, Sunderbans, Eastern and Western Ghats. Such biodiversity hotspots are rich in medicinal floras, thus protection of the indigenous plant species along with collection of raw drugs need to be regulated for sustainable development of natural resources. **Ex-situ conservation** involves setting up of gene banks, herbal botanical gardens, seed banks, drug repositories, nurseries, etc. so that the endangered species can be raised again. The **Department of Biotechnology, Government of India** is working in this line and has taken various initiatives to establish gene banks.

In this regard, loss of traditional knowledge is always felt. Indigenous communities have a culture of worshipping sacred groves which is rich in plant diversity.

They know the ideal growth conditions and micro niches needed for these species to thrive. Even, tribal communities cultivate many medicinal plants for their personal use. For the large scale cultivation of such plants, there is a lot to learn from these communities. Thus, it becomes extremely important to involve tribal people, indigenous habitants in conservation programmes and to document their ethnobotanical knowledge to keep this living tradition alive.

In India, mainly three Acts cover medicinal plant issues at present. They are the **Indian Forest Act, 1927,** the **Forest Conservation Act, 1980** and the **Wildlife Protection Act, 1972**. These acts do not cover much under their domain with respect to import, export, cultivation and trade related issues. Also, they focus on plant and animal resources as a whole but there is no separate law in our country that focuses on the conservation of medicinal flora in particular. The export and import of medicinal flora in India is governed according to **Convention on International Trade in Endangered Species of Wild Fauna and Flora, 1975** commonly known as **CITES**. Being an international law CITES does not cover a vast flora which is country specific. Beside CITES, **International Union for the Conservation of Nature and Natural Resources (IUCN)** is another international organization that works in the field of nature conservation and sustainable use of natural resources. It is involved in data gathering and analysis, research, field projects, advocacy and education. In India, the **Drugs and Cosmetics Rules, 1940** defines ASU drugs; regulate their trade, misbranding, adulteration or substitution and spurious drugs use in any Herbal or Polyherbal ASU formulation. Often, local vendors and manufacturers escape such laws to promote their brand and make profit.

Unregulated harvesting of medicinal plants always takes place outside the protected areas. All over the world, the species of medicinal floras that are banned for export are due to their endangered status in the wild and not because of the medicinal value they hold. There is a need of urgent national level policy for the consolidated effort towards the conservation of medicinal plants. Policies must be formulated keeping in mind the various user groups of medicinal plants. It should advocate in-situ and ex-situ conservation both. The policy should review all existing institutions working in this domain and also build new ones. Most importantly, the policy needs to take into account the legal and regulatory mechanisms related to medicinal plants.

Several efforts are afoot towards the formulation of such policies but we have yet to see something more concrete in this direction.

6. Forensic implications of herbal drugs

A simple understanding about forensics is that anything which is illegal and unregulated by the laws, directly or indirectly tends to harm individuals automatically comes under the purview of Forensic Science.

With the growing trend in medical tourism, most of the western countries have travelers that travel in Asian and East Asian countries for the purpose of taking natural treatment and remedies. Such wellness centres offers a variety of herbal medications for the sale. Patients visiting such centres wrongly believe that they are being treated by natural therapy without getting any harmful chemicals and drugs administered in their body. But how much regulated such centres and their medications are, pose a serious concern. There have been a number of studies that show the adulteration and the substitution in herbal drugs as it is not much regulated by the Pharmacopeia as compared to the allopathic medicines that have strict chemical compositions and are registered under the **Drug and Cosmetic Acts** of different countries [27, 28].

The second most alarming concern about the consumption of adulterated herbal medicines is the **Herb-Drug interaction**. The contaminated herbal

preparations can have anything ranging from heavy metals to pesticides in them that may show antagonistic effects upon interacting with chemical drugs consumed by the patient. Usually patients do not share their history of consumption of herbal drugs as they think it to be natural and posing no side-effects and also there is no such prescription associated with such herbal supplements. The online websites are flooded with such nutraceuticals, dietary supplements and other herbal powder that claim relief and other health associated benefits but neither such companies nor such herbal supplements are often registered under any government regulating agency [29].

Third serious issue is the addition of prescription drugs in the herbal supplements to enhance their efficacy, as this may cause serious issues in the person having allergies to certain compounds. The most common example of such adulterations is the addition of steroidal drugs. Such medications can be contraindicated in a person consuming non-steroidal, anti-inflammatory agents [30].

Fourthly, there is sometimes very high and potentially lethal concentrations of metals are present in these unregulated herbal drugs. When there is an unexpected death occurs, the investigating officers rigorously document all the medications, prescriptions taken by the deceased and found at the crime scene as the medico-legal experts believe the cause of death to the overdose of such prescriptions but unfortunately this is not the case with the natural herbal medicines. Also, there is near to impossible chances of detection of such medicines during autopsy as these medicines are generally consumed by the patient over the long period of time [30].

There have been reports of Lead encephalopathy and acute lead and mercury poisoning in patients consuming herbal drugs. Roger W. Byard in his publication on 'Potential Forensic Significance of Traditional Herbal Medicines' gave examples of such toxicity in which 'a 5- year old boy who had been treated with 'Tibetan Herbal vitamins' and had ingested a total of approximately 63 g of lead over 4 years. He has given another example of a case of a 5-year-old boy with bilateral retinoblastoma whose parents relied on to a traditional remedy that caused him arsenic poisoning.' The issues quoted in the above two cases can be summarized as 'non-medically qualified healers, lack of product standards, undeclared ingredients, nondisclosure of usage and long-term medication' [29].

7. What can be done: a way forward

In the light of the above stated issues, there are a number of measures that can be taken at the country as well as local level for improving the Herbal drug sector and enhancing the development of a more effective trade in medicinal plants and their products in developing countries [16].

a. **Improvement of land resources:** in order to guarantee an uninterrupted supply of raw drug, there must be provisions for organized and protected cultivable land that ensures regular supply.

b. **Reduction in intermediaries:** there are a number of middlemen and intermediaries involved in the market chain of herbal medicines that are needed to be reduced to avoid malfunctions and enhance the profit of primary beneficiaries like farmers and cultivators.

c. **Research and development:** trade and development in any country can grow with more and more investment of GDP in research and development. Research

and development on selected species, their active constituents, processing and preservation is highly needed in developing countries like India. Not only this, but R&D has a major role to play in identification and detection of adulteration in traded herbal products. Thus this area needs a lot of development.

d. **Legal provisions:** for a healthy competition in market, high quality supply of planting materials, quality control systems, improved cultivation and encouragement of investments in new technologies, there should be strict laws and regulatory provisions that guides in these areas.

e. **Value addition:** through processing, and improved marketing of the medicinal plants, value should be added to this industry so that the benefits of the expanded interest in medicinal plants be more equitably shared.

f. **Development of market chain:** a holistic approach needs to be adopted for the promotion of trade. Specific interventions which only target the collectors are insufficient. The organic nature of the produce should be explored and capitalized on for export marketing.

g. **Post-collection handling:** improvements are needed in the areas of post-collection handling, value addition and product presentation.

h. **Sustainable development:** developing countries like India should aim to cultivate their resources in a sustainable manner so that judicious use of resources is ensured.

i. **In-depth approach:** a more in-depth approach should be undertaken to clarify market issues, and consider more effective solutions. Case studies of successful marketing approaches being used may assist other organizations or countries. Products which would be most amenable to sustainable commercial development and industrial processing in the supplying countries must be identified.

j. **Awareness and knowledge:** there should be awareness programmes conducted at intervals for the consumers to educate them about what they consume, how they can decide which product is good for their health, how they can detect in a simple way about the authenticity of herbal drugs while purchasing.

k. **Standardization and quality assurance:** regulating agencies must codify certain standards and parameters that must be fulfilled by the product in order to be sold in the market and the ones that do not qualify those standards should be eliminated at once.

l. **Forensic aid:** whenever any illegal trade, import or export of herbal drugs comes in knowledge of the officials, there is no separate wing of government that tackle such issues. Every country must recognize the need of Forensic intervention in cases of Herbal drugs. There should be well-defined laboratories under forensic department that can analyze the trafficked herbal drug, authenticate it, trace its source and find adulterants and substitutes present in it. Not only trafficked drugs but the drugs from the local market should also be taken into account. Fraud is always a crime whether it is related to accounts/documents/funds which causes monetary loss or related to products sold in the market that causes physical harm.

8. Scientific practices

Unlike conventional Allopathic and Homeopathic drugs that are usually prepared from synthetic, chemically pure materials by means of reproducible manufacturing techniques and procedures, ASU drugs may vary in composition and properties a lot [31]. Thus, in case of ASU drugs also, it is a prerequisite to ensure the reproducibility of batches of medicines by its correct identification and quality assurance so that its safety and efficacy can be maintained [32, 33]. Adulteration, substitution, counterfeiting and usage of inferior quality of drugs often result in degradation of clinical effects of ASU drugs. This makes authentication a crucial step in successful and reliable applications. In cases where there is availability of similar looking drugs or drugs of same chemical nature, authentication becomes more crucial to avoid even any chance adulteration with other varieties of drug. Using a wrong drug may result in worsening the condition or may act as antagonist [34]. Evaluation has become even more difficult when several different individual species were powdered and mixed together in a proprietary medicine [35]. It is also important to take care of each step of harvest, storage, processing and formulation because it has a huge impact on the final quality of the product. Thus, to ensure the optimal safety and efficacy of these products, quality assurance in manufacturing and storage is a must. Also, these control measures are critical for the pharmacological and toxicological evaluation of the ASU drugs. Authentication of ASU drugs involve following techniques:

8.1 Taxonomic identification

In this method, the plant at its source is determined based on its scientific binomial nomenclature, i.e. genus and species determination. This gives the information about the botanical origin of the plant. Before designating the plant to its taxonomic class, its binomial name, vernacular names, site of collection of plant material, details of collector, habitat, season of collection, flowering, altitude and part collected, etc. are required [36].

8.2 Herbarium voucher sample

The sample of collected material should be authenticated by an expert in the field and kept as a voucher sample in a herbarium or a research institute for future references [36].

8.3 Macroscopic method

Macroscopic examination of botanical materials involve examination of certain macroscopic properties also known as organoleptic properties like shape, size, colour, texture, surface characteristics, fracture characteristics, odour, taste, etc. [37].

8.4 Microscopic method

Microscopy is a technique that is used to determine the structural, cellular and internal tissue features of plant material. It helps in identification and differentiation between two herbals that are similar in morphology [38, 39]. This is the commonly used technique, convenient, quick and non-destructive [40]. Microscopy involves varied techniques like compound, bright field, dark field, fluorescence and electron microscopy (Scanning Electron Microscopy known as SEM and Transmission Electron Microscopy known as TEM) that offer different features based upon the requirement.

8.5 Physicochemical methods

Physicochemical parameters include moisture content, total ash content, water soluble ash, acid insoluble ash and sulphated ash. These values of the individual drugs can be compared with the standard values of Indian pharmacopeia and thus their identity can be ascertained [41].

8.6 Spectral methods

Spectral methods like infrared (IR) spectroscopy and ultraviolet- visible (UV–Vis) spectroscopic methods are extremely helpful structural elucidation of active constituents derived from botanical material. Not only structural elucidation but also its fingerprinting can be done by these techniques. They offer comparison between the natural and the synthetic compounds. Also, both qualitative and quantitative analysis can be done. Such techniques when integrated with chemometric tools like Principal Component Analysis (PCA) that help in handling multivariate data without prior knowledge about the study samples, becomes a boon [42, 43]. As chemometric analysis of spectral data does not require any chemical treatment, the method offers a rapid and simple analysis [44].

8.7 Chromatographic methods

High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Thin Layer Chromatography (TLC) are the most commonly used analytical techniques for the profiling of herbal products [45]. Beside these, gas chromatography (GC) can be used for the analysis of volatile components in herbal medicines [46].

8.7.1 Thin layer chromatography (TLC)

TLC is the earliest of chromatographic methods employed for the separation of plant constituents. As the herbal extract is a combination of hundreds of components that are responsible for its activity, it is very important to separate those components and study each of them individually. TLC provides first characteristic fingerprints of herbs. The main advantages of using TLC to develop the fingerprint or chemical profile of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity, simple sample preparation and its economy. Thus TLC is a convenient method to determine the quality and possible adulteration of herbal products [47].

8.7.2 High performance liquid chromatography (HPLC)

After TLC, HPLC has become the most popular method for the analysis of herbal medicines. Though it is more complex than TLC, but still easier to learn and use and is not limited by the volatility or stability of the sample compound as is the case with GC. In general HPLC can be used to analyze almost all the compounds in herbal medicines [48]. HPLC can be linked to various detectors like UV detector, Refractive index detector and Mass Spectrometric (MS) detector that can help in knowing the exact chemical makeup of the components.

8.7.3 Gas chromatography (GC)

Volatile oils and other thermally unstable components that cannot be detected by HPLC technique can be detected by GC. The extraction of the volatile compounds or oils is relatively simple and can be standardized and the components can

be readily identified using GC–MS analysis. The advantages of GC lie in its high sensitivity of detection for almost all the volatile chemical compounds [46].

8.8 Chemical fingerprinting

A chemical fingerprinting is a unique pattern just like human fingerprints which is unique for every individual, which indicates the multiple chemical markers within a sample [49]. The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents, or group of constituents of herbal medicinal product which are of interest, regardless whether they possess any therapeutic activity [50]. Such chemical markers are critical in identification and authentication of the plants from which they are derived. The quantity of a chemical marker is proportional to the quality of herbal medicine. The study of chemical markers is applicable to many research areas, including authentication of genuine species, search for new resources or substitutes of raw materials, optimization of extraction and purification methods, structure elucidation and purity determination.

8.9 Molecular markers

Molecular markers are the constituents that include primary and secondary metabolites and other macromolecules like nucleic acids. DNA as a marker can be a reliable instrument as it is in cases of disputed identity of human beings, in establishing the genetic makeup and polymorphisms of each species [51]. The main advantage associated with DNA is that, it can be extracted from both fresh and dried organic tissue of the botanical material and thus is not restricted by the physiological nature of the sample to be assessed [52, 53]. Various types of DNA based molecular techniques such as hybridization based methods, polymerase chain reaction (PCR) based methods and sequencing based methods are utilized to evaluate DNA polymorphisms [54–56].

9. Conclusion

This chapter has provided an overview of trends and conditions relating to medicinal plants, their production, markets and malpractices. It is never possible to include each and every detail, data and analysis related to medicinal plants in a single chapter. Thus, the chapter is initial effort to increase awareness of both the potentials and problems associated with medicinal plants and its industry. Substitution of herbs can never be eliminated as with more and more dependence on ASU drugs, more than 300 medicinal plants have become red listed. So, in order to achieve constant supply of botanical drugs, experts must find suitable substitute in a regulated manner. Substitution in a well-defined manner may provide a greater scope for the physicians to utilize herbs that are easily available, cost effective and most appropriate for the clinical condition. The need of substitution should always be separated with the intention to substitute and adulterate in order to make more profits. Not all adulterations are intentional. Sometimes it is the lack of awareness of the suppliers about the spurious materials, their toxic effects, confusion in name, non-availability and lack of knowledge about authentic plant may also result in adulteration and substitution. WHO, in its publication on quality standards for medicinal plant materials, recommends rejecting any batch of raw material, which has more than 5% of any other plant part of the same plant (e.g. stem in leaf drugs), never the less if they are derived from the authentic plant. Based on these standards, adulteration whether, intentional or unintentional, should be rejected. In order to

detect such adulterations, technologies have a crucial role to play. The detection of chemical markers is always a good choice in such cases. According to EMEA these chemical markers have nothing to do with the therapeutic activity of the drug; they help in chemical fingerprinting of the botanicals to authenticate its source. Nowadays, Ayurvedic drug industries follow high quality standards using modern techniques and instruments to maintain their quality. Thus, suppliers and traders should also be educated about these parameters to avoid any chance adulteration. Beside all these, there should be strict regulatory laws to govern the quality standards and trade in medicinal flora. Whenever there is any fraud or illegal practice, Forensic Science has always a role to play. Herbal drugs forensic is that only branch of Forensic Science that deals with all such issues.

> *'We can't talk about our own health without understanding our place in our environment, because in order to fulfil our potential we have to live in the context of our surroundings. We have to know our place in the ecosystem of which we are a part, and this means living 'consciously': being aware of nature and how it affects us and how we, in turn, affect nature'.*

A quote from Sebastian Pole's 'Discovering the True You with Ayurveda: How to Nourish, Rejuvenate, and Transform Your Life', very well explains the importance of awareness, be it our surrounding, our body, our food or our medicine. Therefore, Herbal drug forensics is one of the most significant emerging fields of forensic science.

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Conflict of interests

The authors declare no conflict of interests.

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