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APPLICATION OF SALIVA AS PHYSICAL EVIDENCE IN FORENSIC PRACTICE

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Abstract: When a crime is committed, often various objects, the so-called physical evidence, remain at the scene of the incident. Their examination helps to reveal the objective truth and is of crucial importance for the outcome of a particular case. Saliva analysis has wide application not only as a diagnostic tool in medical and dental practice, but also in the work of the bodies taking part in the pre-litigation and litigation proceedings. This article aims to outline the areas of saliva application in practice, summarizing its use in the different contexts of forensic medicine and forensic odontology.

Keywords: Physical evidence, saliva, forensic medicine, forensic odontology

INTRODUCTION

Forensic medicine is a medical science of applied nature, which studies, develops and helps to resolve a number of medical and biological issues arising in connection with the work of the judicial authorities. When a crime is committed, often various objects which have been used in the doing of an act or on which there are traces of a crime, the so-called physical evidence, remain at the scene of the incident. These "silent witnesses" can be various weapons, labor tools, random objects, biological traces, documents, etc. [1]. Their examination helps to reveal the objective truth and is of crucial importance for the outcome of a particular case. Saliva analysis is widely applied in crime detection, drug abuse, alcohol abuse, intoxications and for many other reasons. Over the last decade, interest in saliva, not only as an object found at the crime scene, but as a diagnostic tool too, has grown both in the medical and the dental practice, and in the work of the bodies taking part in the pre-litigation and litigation proceedings.

This article aims to outline the areas of saliva application in practice, summarizing its use in the different of contexts of forensics medicine and forensic odontology. For this purpose, a selective search and review of scientific literature was conducted in PubMed databases and by means of Google Scholar search engine, using the following key terms: "physical evidence", "saliva", "forensic medicine" and "forensic odontology". The search was limited to primary, secondary and tertiary scientific literature published between 1974 and 2021, and the selected publications were included on the basis of their relevance and significance to practice.

General aspects and characteristics of saliva

The parasympathetic part of the nervous system stimulates salivary secretion leading to the release of the neurotransmitter acetylcholine, which binds itself to the muscarinic receptors, predominantly type 3. These receptors cause the activation of phospholipase C, which creates the secondary mediator inositol triphosphate (IP3). IP3 migrates to receptors in the endoplasmic reticulum which serves as an intracellular site for calcium storage. Once IP3 binds to this receptor, ionized calcium is released [2-5]. This controlled increase of cytosolic calcium activates the potassium and chloride channels, forming a salt gradient, which leads to the release of salivary secretions [2-4]. Salivary glands are divided into two main groups: the major glands consisting of three pairs of major salivary glands (parotid, submandibular, and sublingual), and a multitude of minor glands which are located in the

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labial, buccal, oral and palatal tissues. They are also classified according to their function - the parotid glands produce serous secretion; the submandibular glands produce serous and mucous secretion, and the sublingual and minor salivary glands produce primarily mucous secretion [2, 4, 6]. The mixture of secretions released by the salivary glands, together with the gingival crevicular fluid, take part in the formation of the so-called "whole saliva". Whole saliva contains non-salivary ingredients, such as desquamated cells, bacteria and their metabolic products, various blood components, etc. [2, 4]. The normal secretory levels of whole saliva vary between 800 ml/24h and 1500 ml/24h with a pH value in the range of 6.0 to 7.0 on average [2]. This pH range is effective for activating the digestive enzyme α-amylase, which is found in serous saliva and catalyzes the breakdown of starch. Saliva contains potassium, sodium, bicarbonate, and chloride ions, which are important for the activation of the taste receptors located on the taste buds. Two major mucins, mucin 1 (MUC1) and mucin 2 (MUC2), play an important role in the lubrication of the mucosal surfaces. Saliva also secretes significant amounts of antimicrobial and immunomodulatory proteins, which contribute to mucosal lubrication and tissue coating, tooth remineralization and buffering. They include histatins, proline-rich proteins, statherins, carbonic anhydrases, secretory IgA (immunoglobulin A), lactoferrin, lactoperoxidase, lysozyme, amylases, and a protein with putative anti-HIV (anti-human immunodeficiency virus) capabilities (secretory leukocyte protease inhibitor or SLPI) [2-4].

Methods for detecting saliva stains

Saliva has a wide range of applications in forensic medicine. It is most often found on used household utensils and kitchenware, discarded cups, straws, butts, various packages, envelopes, stamps, etc. [1, 7], but it can also be found in cases of bites in animal attacks or in acts committed for the purpose of robbery, sexual violence, murder and other criminal offenses [1, 2, 8, 9]. Its analysis can be used to identify the perpetrator of a bite, a suspect or a victim of violence or disaster. The main work process in identification includes visual examination, orientation analysis, confirmatory (evidential) analysis, determination of the species, group and sex affiliation, and DNA (deoxyribonucleic acid) typing.

Saliva is a transparent liquid and after being deposited on objects at the crime scene or on the skin of a victim/suspect, it becomes practically "invisible" to the human eye, so its detection is the first step of the identification process [10]. Saliva stains in dry state are relatively stable and if environmental conditions are favorable, saliva can be detected months or years after being deposited. It should be taken into consideration, however, that heat, moisture, sunlight, surface contaminants and other factors such as various laundry detergents can accelerate DNA degradation or remove all traces of dried saliva stains [2, 9, 11]. In their studies, Ohta and Ohmura have established that the acid in citrus fruits or some soft drinks, as well as positively charged Fe3+ and Mg2+ ions (contained in blood stains and in some foods and medicines) can inhibit the enzymatic activity of α-amylase [12, 13]. Saliva detection methods have limitations and variable sensitivity depending on the age of the stain and the amount of deposits [7]. Saliva establishment is most often done by detecting ptyalin (alpha amylase), an enzyme characteristic for saliva [1]. Another commonly used enzyme is alkaline phosphatase. Salts such as nitrate and thiocyanate are also used to detect saliva [14]. In his studies, Auvdel compares the use of argon ion lasers, sources of ultraviolet light [15] and high-intensity quartz arc tubes [16] to detect body secretions, including saliva, discussing the advantages and disadvantages of these methods. When using argon ion lasers and quartz arc tubes, saliva is presented in the form of white stains with soft edges, less intensive than other stains [7]. Saliva spots appear bluish-white under ultraviolet light, but it should be taken into account that this is due to the DNA degradation of the sample [17]. In addition to the methods mentioned above, the indispensable aromatic amino acid, tryptophan, present in salivary α-amylase, gives a characteristic emission spectrum in fluorescent spectroscopy, thus showing good sensitivity in detecting dried saliva stains on the skin [14]. Methods using Raman spectroscopy [17] or Fourier-transform infrared spectroscopy [18], have experimentally shown positive properties as non-destructive methods for sample identification [19]. A promising new marker for saliva identification is the detection of oral streptococci (S. salivarius, S. sanguinis and S. mutans) [20, 21], which are not found in other biological fluids (semen, urine, vaginal fluid) or on the skin surface, by using polymerase chain reaction (PCR) alone [22] or in combination with an immunochromatographic strip [21]. In recent years, mRNA (messenger ribonucleic acid) profiling by reverse transcription-polymerase chain reaction (RT-PCR) has been reported as another new approach to identify body fluids. Messenger RNA is the intermediate molecular compound between the

genomic DNA and the expressed protein. Apart from being compatible with the current procedures based on DNA analysis, the mRNA-based approach offers other advantages over conventional methods for identifying body fluids as well [9, 23], among which are: greater specificity and timeliness, saving time and numbers of samples, etc. Sakurada K. *et al.* have described the use of real-time RT-PCR analysis, using mRNA expression levels of the saliva-specific proteins statherin (STATH) and histatin (HTN3), which show high specificity to this body fluid [19]. They have examined the HTN3 stability in saliva stains under different environmental conditions over time and the results of the study have shown that the mRNA degradation in the stains has strongly been affected by moisture and light. Nevertheless, using this method, they have detected saliva in an about 6-year-old stain, stored in a place with dry conditions and no access of light [19]. Biological fluids, found in different forensic cases (saliva, blood, sweat, semen, vaginal secretions, etc.) can sometimes be mixed samples and consist of cells from multiple tissues. Such circumstances are often observed in sexual offences. The collection of genes expressed in a body fluid or tissue is known under the term "multicellular transcriptome". The patterns of mRNA expression can provide information for specific cells and tissues which can be used to positively identify the source of the tissue [2, 9]. In addition, methods focused on microRNA [24, 25] and DNA methylation [26, 27] have actively been tested in saliva identification, but further research is still needed to be done before these methods can be applied in practice.

After establishing the stain as being saliva, the sample can be collected either by using the technique of rubbing for several seconds (10 sec) with a single wet cotton swab soaked in distilled water or by using wet filter paper laid passively on the skin. In addition, the double swab technique could be applied by using a single wet cotton swab followed by a dry cotton swab, in combination with light pressure and circular motions, again for a few seconds (10 sec) [13, 28]. It has been established that the latter technique, used in the studies of Sweet and al., provides better sample quality, which is associated with the rehydration of the traces left [29]. Saliva sampling kits are also available in commercial retailing today when DNA testing is required [30]. By using serological and cellular analysis, the saliva extracted from the object of deposition can be examined so as to determine its species and group affiliation. In cases of animal attacks and the presence of bites on the victim's body, the species affiliation of the established saliva can be determined by the species-specific genetic profile of the particular animal [31]. A certain "secretor's ability" (Se or se) can be established in the person's saliva, and the finding of epithelial cells from the oral mucosa can, in some cases, also determine the sex affiliation due to the existence of the sex chromatin X or Y in the nuclei of these cells [1]. Fletcher *et al.* describe an enzyme-linked immunoassay using a monoclonal antibody based on the presence of salivary immunoglobulin A to identify the species [32]. Other methods used to identify an unknown saliva sample are crossed electrophoresis and double gel diffusion techniques [7].

Sample identification processes involve DNA extraction from saliva by phenol-chloroform or another standard method, and DNA sample amplification by the polymerase chain reaction (PCR) method, using short tandem repeats (STR) with a length of the main motif of 1 to 6 nucleotides [1]. At the present stage of forensic investigations, DNA analysis of single-locus microsatellite markers, having an exactly established chromosomal localization only in a specific region of the genome, is used [1]. Due to the presence of a significant number of buccal epithelial cells in the saliva containing nuclei, it is an excellent source of DNA, from which a relatively easy genetic profile can be obtained [9, 26]. Except for identical twins, DNA profiling is unique for every person. Such an analysis requires approximately 0.5 – 1.0 ng of DNA, which is about 80 – 160 cells [9, 33]. In addition to chromosomal (genomic) DNA, which is inherited from both the mother and the father, saliva derived cells also contain mtDNA (mitochondrial DNA), which is inherited only from the mother [34, 35]. The main advantage of mtDNA is that, on the one hand, it is found in the mitochondria of the cells and is in a large number of copies [35], and, on the other, when there are no close relatives, it allows distant maternal relatives to be used for comparison in identification [36].

Saliva analysis in psychoactive substances abuse

In modern practice, blood and urine are the most commonly used biological fluids to determine the presence of alcohol and other psychoactive substances, including narcotics, in the human body, in both living and dead subjects of study. Blood sampling, however, is an invasive manipulation associated with causing pain, which carries the risk of complications and must be performed by a doctor. In addition, even non-invasive urine sampling is disputable in view of the invasion of

privacy, especially if it is advisable to monitor it directly to prevent adulteration or substitution of the sample [37]. Saliva is gaining popularity as a test object alternative to blood and urine due to the fast, painless and non-invasive nature of its collection and the possibility of monitored sampling [7, 8, 37, 38]. Most drugs enter saliva through simple passive diffusion which is characterized by a transfer of drug molecules down a concentration gradient with no energy expenditure [39, 40]. The concentration of a detectable substance in saliva is usually proportional to the free fraction of the same substance present in the plasma. Therefore, this measurement makes it possible to compare the concentration of the substance and its pharmacological effects on the individual. There are various methods by which saliva samples can be provided, most often using the method of spitting [8, 41] and the swabbing technique. Various substances can be identified in saliva such as ethyl alcohol, opiates and opioids, stimulants (cocaine, amphetamines, methamphetamines), ecstasy, phencyclidine, prescription drugs (barbiturates, benzodiazepines), etc. [42]. Saliva can also be used to detect the recent use of marijuana (4 hours after smoking) through identifying the main psychoactive component of the drug – tetrahydrocannabinol (THC) [7]. Saliva samples can be examined indicatively by immunoanalytical methods, followed by the confirmatory method of gas chromatography with mass-spectral detection [1, 43, 44], a gold standard in modern chemical and toxicological studies.

Application of saliva in diagnosing diseases and conditions

Saliva is the main biological environment in which the processes in the oral cavity take place [38, 45]. Diet, different diseases (obesity, diabetes, gout) and medication intake in cases of polypragmasia can change the amount, buffering properties of saliva and the virulence of the oral microbiota, resulting in a wide range of disorders and conditions that can be detected when examining this sample [8, 46]. Saliva is used for the quantitative determination of steroid hormones such as cortisol, cortisone and testosterone as well, because it is accepted that the salivary levels of these steroids reflect the free unbound circulating fraction [47]. For this reason, it is also used in behavioral analysis as studies have shown that these hormones, depending on their level, determine different behavior and way of thinking of the person. High testosterone levels are found in people who show a ruder behavior, less consistency in their actions and individual resentment [48]. Similar data are available for low cortisol levels, which are associated with more violent criminal behavior also [49]. Saliva can likewise act as an analytical agent in cases of heavy metal poisoning, and cadmium (Cd) and lead (Pb) can be identified by using atomic absorption spectroscopy or mass spectroscopy [13]. Furthermore, leaks from dental materials such as mercury, nickel, zinc, etc. can also be detected by saliva analysis in dental practice.

In conclusion, it is common knowledge that any commission of a crime involves a transfer of different types of biological material unilaterally or bilaterally between the victim and the perpetrator, and between them and the various objects at the crime scene, including the weapon of the act. Saliva, like the other biological fluids (hairs, blood, sweat, tear secretion, vaginal secretion, urine, etc.) plays a crucial role in the forensic medical examination of physical evidence in incriminating the perpetrator, establishing the manner of committing the crime and clarifying a number of issues that cannot be resolved in any other way than by using forensic medical means. Despite its usefulness and advantages, saliva analysis is difficult to perform because saliva is unstable. Salivary proteins are easily degraded due to the proteolytic activity of the enzymes released by cells and various microbes. Saliva stains are not always detected, because some substances on the surface on which the traces have been deposited may make it undetectable for indicative study methods. In recent years, the number of samples of physical evidence sent for testing in laboratories has increased, as has the need to analyze very small quantities of them, which are often old and poorly preserved. Therefore, identifying body fluids by faster and more accurate methods is absolutely crucial. In recent years, immunochromatographic methods have been introduced in the identification process and molecular-biological ones have also been developed in order to determine new markers and their applicability in practice. The use of saliva in the detection of drug use by drivers has become increasingly popular in recent years. Saliva is also an analytical agent in cases of poisoning as it reflects the resulting ionic imbalance. Its application is huge and in many areas, and only the future can show what else lies behind every drop of saliva in the mouth.

Conflict of interest

The authors declare that they have no conflict of interest.

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