Chapter

Samples Used in Molecular Autopsy: An Update

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Abstract

Molecular autopsy makes it possible to identify the genetic alteration responsible for an inherited arrhythmogenic disease, main suspected cause of sudden death in cases that remain unexplained after a complete medico-legal autopsy. By using next-generation sequencing technology, a massive genetic study can be carried out that identifies a rare variant classified as potentially pathogenic in up to 25% of sudden death cases in the young population. To carry out a post-mortem genetic study, it is necessary to have samples in suitable conservation conditions. Our chapter focuses on the type of samples that are used today in massively parallel genetic analyses.

Keywords: samples, molecular autopsy, genetics, tissue, paraffin, blood

1. Introduction

Currently, in nearly 5% of all cases after comprehensive forensic autopsy no definite cause of death is found, allowing for the definition of "negative autopsy" [1]. When autopsy fails to find the cause of decease, sudden death can be called "unexplained" (SUD) [2]. In SUD cases, especially in those younger than 35 years, inherited arrhythmogenic syndrome (IAS), are frequent cause of death and thus, sudden cardiac death (SCD) should always be suspected [3]. All IAS are of genetic origin and therefore, family members can be carriers of pathogenic genetic alterations, and in consequence, be at risk for SUD. The first manifestation of any of IAS may be a lethal arrhythmogenic episode, highlighting early identification of genetic carriers and allowing the adoption of preventive personalized therapeutic measures [4]. In 2001, a post-mortem genetic analysis or post-mortem molecular analysis (also called "molecular autopsy") was firstly proposed [5] as a fundamental tool in order to unravel the genetic origin of an IAS as cause of SUD [2]. To date, molecular autopsy has become a complement to autopsies process in the current forensic area. Molecular autopsy has been shown to be a reliable diagnostic tool during a comprehensive forensic investigation of SUDs and may have important implications for the

first-degree relatives of the victim leading to further analyses to predict and prevent the risk of life-threatening events [6].

2. Genetic analysis

First genetic approach in molecular autopsy was Sanger technology which has played a significant role allowing the identification of the first genes associated with IAS related to SCD. Although, the Sanger sequencing technique has played an important role in the history of molecular genetics and has been very useful in the study of SCD for many years, at present, its use in clinical practice has been reduced [7]. For years, Sanger sequencing was the gold standard for investigating SUD cases until it was replaced by second-generation high-throughput techniques, called Next generation sequencing (NGS) [8]. This is due to the fact that Sanger technology only allows the study of a limited number of genes and at a high cost, compared to massive next-generation sequencing techniques. However, it remains the gold standard technique for variant confirmation, especially for small deletions and insertions. Nowadays available NGS technologies allow a rapid and cost-effective genetic analysis of numerous genes (even whole exome -WES- and genome -WGS- sequencing). NGS has enabled the identification of more than 2400 new disease-associated genes and more than 150 new genetic diseases [9].

Finally, third-generation sequencing technologies, also known as single-molecule sequencing, allow for the direct sequencing of single DNA molecules without the need for amplification or fragmentation. Although this technology promises to improve the range of detection of causal variants in a wide range of pathologies, since it has some advantages such as the possibility of studying structural variants and repetitive elements, its implementation in clinical practice has not yet materialized. It is partly due to certain limitations such as the high cost compared to NGS, the need for a more complex bioinformatics analysis, and perhaps the most important limitation in our field, the fact that it requires fresh material to obtain ultralong DNA of high molecular weight, which can be a great challenge in post-mortem analysis [10, 11].

3. NGS applicability to SUD

The routinary genetic study carried out using NGS technology in SUD consist of analyzing the main genes currently associated with IAS, either by amplification and sequencing of gene panels, or by performing WES and subsequent filtering of the genes of interest [12–14]. The number of genes analyzed increase as progress is made in the field of IAS [15]. In young population, molecular autopsy using NGS reveals a definite pathogenic genetic alteration responsible of an IAS in near 20% of cases [16–22]. It is important to remark that genetic alterations identified in IAS are genetic defects in ion channels expressed in heart as well as in brain, therefore being the main cause of sudden death episodes during epilepsy (Sudden Unexpected Death in Epilepsy, SUDEP) [23–25].

After molecular autopsy with a positive genetic diagnosis, due to other family members could harbor the same genetic variant and, thus at risk of IAS, a clinical translation of genetic results should be performed [4]. In such cases, first-degree relatives of a SUD victim should undergo a multidisciplinary evaluation including clinical examination and genetic analysis [6, 26]. To date, main challenge in clinical

translation of genetic data is the interpretation of large part of variants identified, remaining of unknown significance (VUS). Firstly, this is due to the stricter classification provided by the American College of Medical Genetics (ACMG) [27]. Moreover, either variants are found in genes with no definite association with any of IAS or available data does not allow a deleterious role of a variant to be assigned. Despite this fact, current clinical guidelines recommend molecular autopsy in SUD cases when the victim is young (< 50 years of age) and/or the circumstances of death and/or the family history support an IAS as the most plausible cause of SUD [4, 6, 28, 29].

4. Samples

Collection of samples for molecular autopsy is a crucial step in the forensic analysis of SUD and is recommended by several guidelines [6, 30]. Suitable sampling in terms of site and of timing is crucial also because of the risk of low-template DNA, i.e., inadequate quality and/or quantity of extracted DNA. Indeed, in IAS, up to 40% of samples are not collected adequately for post-mortem genetic study preventing an appropriate analysis [31]. In an exploratory study, fresh blood and frozen blood were reported as the most common types of post mortem samples. In addition, fresh blood and frozen blood had the highest number of successful DNA extractions, but blood spot cards, frozen liver, and frozen heart tissue were also reported to have successful DNA extractions [32]. Recently, a consensus focused on post-mortem study of SCD cases was published, recommending blood as optimal sample for molecular autopsy despite other kind of samples can be also used if appropriate collection and storage, such as fresh/frozen tissues or formalin-fixed and paraffin-embedded (FFPE) tissues [14].

4.1 Blood

This is the easier approach to obtain and storage a post-mortem sample in order to perform a molecular autopsy. The sample will be preferably extracted from the subclavian vasculature by puncture prior to opening the thoracic cavity. If not possible, the thoracic and abdominal cavities will be opened, removing the visceral blockage and puncturing the right atrium afterwards for blood collection [33]. It is recommended to collect 3–5 ml of peripheral or intracardiac blood less than 48 hours post-mortem in Ethylene Diamine Tetra Acetic acid (EDTA) tubes (**Figures 1** and **2A**). If sample is collected more than 48 hours post-mortem, the degradation of DNA increases progressively despite conservation of body at cold temperature. This degradation may impede a proper DNA extraction and NGS analysis. EDTA tubs should be also store at cold temperature (4–8°C) but if DNA will be extracted during first 48 hours after collection, tubes can be retained at room temperature (no more than 20°C) [34]. If DNA extraction will be programmed more than 2 days after collection, it is highly recommended store tubes at 4°C (maximum 2–4 weeks) [34]. More than one month after extraction, DNA may be progressively degraded, so freezing at a minimum of −20 to −80°C is recommended [14, 35]. However, it is important to note that freezing the EDTA tube should be avoided as much as possible, as the freezing and thawing process damages the DNA structure. In this situation, and in order to preserve DNA integrity, thawing process should be performed progressively (−20 to 4–8°C for at least 1–2 days, and then to room temperature). DNA extraction should be performed at routine room temperature in laboratories (around 15°C). This is the

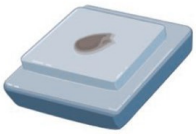
Blood	***Dried blood spot (DBS)***	***Fresh/frozen tissue***	***Saliva***	***Formalin-fixed and paraffin-embedded***
				
• 4-8°C (max. 48h) • 4°C (max. 2-4 weeks) • -20°C (more than one month)	• -4°C- room temperature (max. 2 weeks). • -20°C (more than 2 weeks).	• 4-8°C (max. 12h). • -20-80°C (more than 12h)	• 37 °C (max. 18 months)	• 37°C (many years)

Figure 1.
Samples used in molecular autopsy analysis and their conservation methods.

optimal protocol to avoid a rapid DNA degradation, which prevents a proper NGS analysis.

4.2 Dried blood spot (DBS)

Most NGS analyses are performed using EDTA-anticoagulated peripheral blood, because of the high quality of DNA extraction obtained from such samples (**Figure 1**). However, collecting, transporting and storing blood in some circumstances can represent a challenge. An alternative may be the use of Dried blood spots (DBS), since NGS techniques require only short fragments of DNA for sequencing, which can be obtained with this collection technique [14, 35, 36]. DBS is an inexpensive method that is easy to handle in all conditions and does not require a trained professional for collection [37]. DBS can be storage at ambient temperature for many months and even years but the quality of DNA extracted could be reduced progressively [38]. Although recent studies support a sequencing yield with DNA from DBS samples, similar to that obtained with DNA samples from peripheral blood, both in the detection of single nucleotide variants, insertions and deletions, as well as copy number variants and mitochondrial heteroplasmy, such studies are limited and their throughput in NGS techniques applied to postmortem analysis of SUD has not been confirmed [39–41].

4.3 Fresh/frozen tissue

Fresh tissue samples from cadaver should be collected for post-mortem genetic testing (**Figures 1** and **2B**). The 2020 APHRS/HRS expert consensus statement recommends that samples of heart (especially if SCD is suspected) and at least one spleen/liver/skeletal muscles sample should always be saved [35]. About 5 g of tissue is optimal to perform a quick DNA extraction (no more than 6–12 hours after extraction and retained at 4–8°C) in order to avoid DNA degradation. Focused on SUD and suspected IAS, tissue should be of heart (optimal from ventricular myocardium). If not analyzed in this time period after extraction, the fresh samples should be stored at freezing or ultra-freezing temperatures (−20 to −80°C) [35]. In this situation, defrosting should be carried out progressively (−80 to −20°C, then to 4–8°C for at least 1–2 days, and finally to room temperature) before DNA extraction to avoid degradation, as mentioned for frozen blood. DNA extraction should be performed at routine room temperature in laboratories (around 15°C).

4.4 Saliva

DNA extraction from saliva for high-quality genetic analysis is widely used for living people and many devices are available for the sample collection (**Figure 1**) [42]. It is a widely used sample in the genetic study of many pathologies given the multiple advantages it presents in terms of sample collection, transport and storage. Saliva can be stored at room temperature for up to 18 months without compromising its quality for genetic analysis [43]. Although in forensic genetics buccal swabs are indicated for identification in recent corpses, sampling cadaveric saliva is usually not recommended, since it is technically challenging and can be biased by post-mortem changes. Other samples with the same or lower extraction complexity may yield better quality DNA samples.

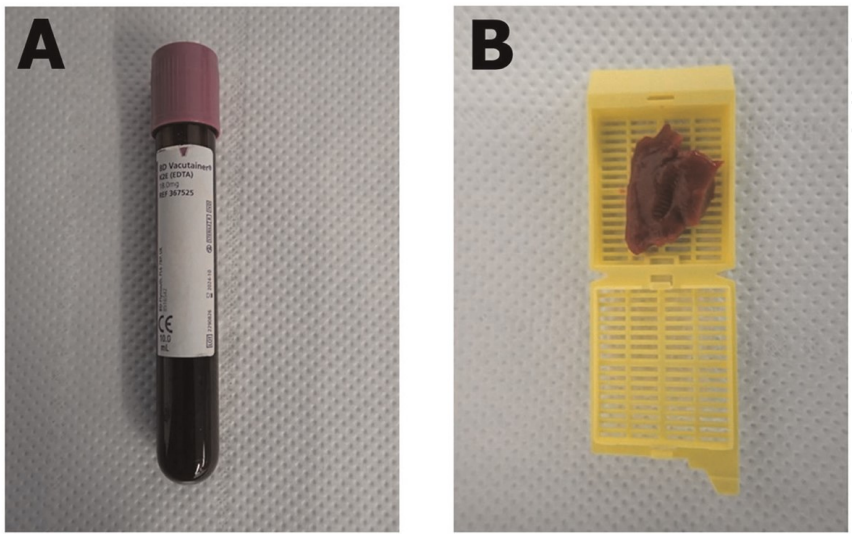


Figure 2.
Real samples mostly used in post-mortem NGS analysis. A. Blood sample. B. Fresh tissue sample.

4.5 Formalin-fixed and paraffin-embedded (FFPE) tissues

Currently, FFPE tissue samples are processed and stored, as part of routine forensic protocol in order to unravel any tissular alteration (**Figure 1**) [44]. In heart FFPE tissue, several alterations such as inflammatory cell infiltration, myocyte apoptosis or any other alteration may clarify the cause of death. Focused on IAS, tissular alterations may confirm the suspected diagnosis. Sometimes, macroscopic study does not reveal any alteration but FFPE analysis identify alterations such as disarray or fibro-fatty infiltration in myocardium, hallmarks of cardiomyopathies [45]. It is possible to extract DNA from FFPE stored for more than 25 years for molecular analysis [46]. These samples represent a suboptimal material for molecular autopsy as storage in formalin has been shown to damage DNA, which is then variable in both quantity and quality [44, 47].

In order to use FFPE for NGS analysis, protocols of tissue fixation and paraffin embedding usually damages the DNA integrity preventing an adequate NGS study from being performed according the recommended protocols [48]. Tissue samples that were formalin-fixed did not have high rates of successful DNA extraction, which is consistent with evidence found in a past study that showed formalin-fixed samples are unreliable for post mortem genetic testing in cases of sudden unexplained death [32]. DNA from FFPE have been considered error prone and unreliable in comprehensive surveillance of SUD-associated genes. Given these shortcomings, the standard autopsy for SUD should include archiving EDTA-preserved blood or frozen tissue to facilitate post mortem genetic testing [49].

However, several studies have been carried out obtaining proper DNA from FFPE heart tissue in IAS but with a wide range of technical variables preventing a standardized use of FFPE for a comprehensive NGS analysis [50, 51]. In order to solve this limitation, in last years, adapting protocols and special kits focused on DNA extraction from FFPE samples has been developed, helping to use this kind of samples, especially in old cases if no other sample available.

5. Ethics considerations

Although in some countries it is not necessary to have a specific and express consent to perform a genetic analysis on the sample of a deceased person, current legislation must be taken into account before performing a molecular autopsy. When identifying a genetic alteration responsible for the pathology or if the results of the studies are of interest to other members of the deceased's family, it is important to take into account ethical concepts to preserve the privacy and protection of the family members' data, as well as the impact on the health of the individuals who may be at risk. In the case of minors or individuals with intellectual disabilities, the parents or guardians/legal representatives are the ones who must make the decision for them. Communication of the results of genetic testing and autopsy to the family should ideally be performed by a multidisciplinary team composed of cardiologists and genetic counselors specialized in cardiovascular genetics at a medical center, in the context of genetic counseling [14].

6. Conclusions

Finding the cause of the death in SUD cases should be considered a public health priority since, especially in young population, these events are mainly due to IAS.

The current standard techniques for postmortem molecular analysis are those focused on panels of known SUD-associated genes, both because it allows the analysis of a large number of genes at low cost, and because it requires a small amount of DNA obtained from a wide variety of samples for analysis. Sampling is a crucial step in molecular autopsy to avoid the risk of low-template DNA and thus to maximize DNA yield. Post-mortem blood and fresh or frozen highly vascularized tissues are optimal sources of DNA, while the recourse to FFPE tissues should be reserved when other strategies are not feasible, since the risk of low-template DNA.

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Conflict of interest

All authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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