Postmortem Genetic Testing for Conventional Autopsy–Negative Sudden Unexplained Death

An Evaluation of Different DNA Extraction Protocols and the Feasibility of Mutational Analysis From Archival Paraffin-Embedded Heart Tissue

Elisa Carturan, PhD,[1] David J. Tester,[2] Brian C. Brost, MD,[2] Cristina Basso, MD, PhD,[1] Gaetano Thiene, MD, FRCP,[1] and Michael J. Ackerman, MD, PhD, FACC[2]*

Key Words: Sudden unexplained death; Genetic testing; RYR2; Catecholaminergic polymorphic ventricular tachycardia; Paraffin-embedded tissue; DNA extraction; Autopsy

DOI: 10.1309/VLA7TT9EQ05FFVN4

Abstract

One third of autopsy-negative sudden unexplained deaths (SUDs) can be attributed to a cardiac channelopathy. Typically, paraffin-embedded tissue (PET) is the only source of DNA available for genetic analyses. We examined different DNA extraction procedures, involving 2 deparaffinization methods, 2 digestion methods, 4 laboratory-based purification methods, and 5 commercial kits. Mutational analysis involving 25 RYR2 *exons was performed on PET DNA from 35 SUD cases to evaluate the feasibility of using PET DNA for genetic testing. With the best PET-DNA extraction method, an average of only two thirds of the region of interest could be evaluated. Although we initially identified 5 missense mutations in 5 of 35 SUD cases, repeated analysis failed to confirm these mutations. DNA from PET should be 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 postmortem genetic testing.*

In developed countries, sudden cardiac death (SCD) is one of the most common causes of death, and, tragically, thousands of people younger than 40 years die suddenly each year. Fortunately, in many cases, the cause and manner of death can be established from a comprehensive medicolegal investigation, including autopsy.[1,2] For nearly half of young victims from 1 to 35 years of age, there are no obvious warning signs, and sudden death often occurs as the sentinel event, thus placing extreme significance on the medicolegal investigation and autopsy to determine the cause and manner of death.[3] A postmortem examination may detect a noncardiac basis for the sudden death such as asthma, epilepsy, or pulmonary embolism. However, SCD is the predominant cause of sudden death in young people, with structural cardiovascular abnormalities often evident at autopsy.[2,4,5]

However, not all SCD has an apparent attributable cause that can be determined at autopsy. It is estimated that as many as 30% of sudden deaths involving previously healthy children, adolescents, and young adults have no identifiable morphologic abnormalities found at autopsy, and the SCD is labeled as conventional autopsy–negative sudden unexplained death (SUD).[1,2,6-8] Potentially lethal and heritable channelopathies such as catecholaminergic polymorphic ventricular tachycardia (CPVT), congenital long QT syndrome (LQTS), congenital short QT syndrome, and Brugada syndrome leave no evidence to be found by a comprehensive medicolegal autopsy, leaving coroners, medical examiners, and forensic pathologists only to speculate that a fatal arrhythmia might lie at the heart of an SUD.[1,9-12]

However, owing to molecular advances, a cardiac channel molecular autopsy may potentially illuminate such a pathogenic mechanism and establish probable cause and manner

for SUD.[13-19] In a series of 49 coroner's cases of conventional autopsy–negative SUD, the cardiac channel molecular autopsy consisting of cardiac ryanodine receptor 2 (*RYR2*) mutational analysis for CPVT and the equivalent of the commercially available LQTS genetic test for *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* elucidated mutations in more than one third of the cases, with 20% linked to CPVT and 15% linked to LQTS.[13-19] Such postmortem genetic testing can be performed readily using "DNA-friendly" autopsy material such as EDTA-preserved blood or fresh frozen tissue.[13-19]

However, the vast majority of medical examiner's offices do not archive tissue in this manner. Instead, owing to ease of storage and transport, archived formalin-fixed, paraffin embedded tissue (FF-PET) is typically the only source of DNA available for procurement. During the past several years, numerous extraction protocols have been reported to give high-yield, quality DNA from FF-PET. Thus, the purposes of this study were to evaluate different DNA extraction procedures to develop the most efficient method for obtaining DNA from archived FF-PET suitable for postmortem mutational analysis and to assess the feasibility and quality of DNA extraction to perform a molecular autopsy for the most common cause of conventional autopsy–negative SUD, namely *RYR2*-mediated CPVT.[20]

Materials and Methods

Samples

FF-PET blocks of autopsy tissue from the myocardium, with different durations of storage (3-22 years), were chosen randomly from an SUD cohort at the Mayo Clinic, Rochester, MN. Each block represented a unique SUD victim. The fixation method and time of tissue sampling were not known. To serve as a positive control sample, 1 block represented a sample from a case shown to harbor a pathogenic *KCNQ1* mutation through a previous analysis using autopsy material from whole blood.

Study Design

This study was performed in 3 consecutive steps: (1) Three different blocks were processed using 26 permutations involving 2 deparaffinization methods, 2 standard protocols proposed for digestion, 4 purification methods, and 5 commercial kits. Following DNA isolation using the various protocols, the quality of the extraction was evaluated by polymerase chain reaction (PCR) amplification of 4 amplicons ranging in size from 199 to 300 base pairs (bp). (2) By using the best extraction methods obtained in step 1, the number of PET blocks analyzed was increased to 11. (3) DNA was extracted from 35 FF-PET blocks with the best method obtained in step 2. A combination of PCR, denaturing high-performance liquid chromatography (DHPLC), and DNA sequencing of the CPVT1-associated cardiac *RYR2* gene was used to assess the ability to perform postmortem genetic testing on FF-PET–derived DNA.

Sample Preparation

One 10-μm-thick section from each FF-PET block was cut using a standard microtome with a fresh disposable blade, and each section was placed in a microcentrifuge tube. The microtome and work areas were cleaned thoroughly with ethanol between specimens to remove debris.

DNA Isolation Protocols

In general, DNA isolation from FF-PET involves 3 unique phases: (1) tissue deparaffinization, (2) tissue digestion, and (3) DNA purification.

Deparaffinization

Xylene, Temperature, and Ethanol.—The tissue section was incubated twice in 1.5 mL of xylene for 15 minutes at 50°C. Subsequently, the tissue sample was rehydrated in decreasing concentrations of alcohol (ethanol, 100%, 95%, and 70% and phosphate-buffered saline for 5 minutes each). To avoid any loss of sample, centrifugation (12,000 rpm, 5 minutes) was performed before each change in solution, with each solution carefully removed using a fine pipette tip. The sample was allowed to air dry at ambient temperature for 15 minutes.

Microwave.—For the microwave method,[20] tissue sections were crushed using a sterile pipette tip in 200 μL of buffer (50 mmol/L of tris(hydroxymethyl)aminomethane hydrochloride [Tris HCl], pH 8; 1 mmol/L of EDTA, pH 8; and 0.05% polysorbate 20). The tube was tightly capped, placed into a microwave oven, and subjected to microwave irradiation at high power (500 W) for 30 to 60 seconds.

After deparaffinization the tissue was disrupted with a mechanical tissue disaggregator (pellet pestle, Sigma, St Louis, MO) and subsequently subjected to 1 of 2 digestion protocols.

Tissue Digestion

Proteinase K–Overnight Digestion.—For the Proteinase K–overnight digestion method,[21] 400 μL of digestion solution (50 mmol/L of Tris HCl, pH 8.5; 1 mmol/L of EDTA; and 0.5% polysorbate 20) and 20 μL of Proteinase K (20 mg/mL) was added to the deparaffinized tissue and incubated at 50°C in a thermomixer set to shake at 100 rpm overnight.

Sonification.—Sonification[22] was performed in Branson Model 2200 (Branson Ultrasonic, Danbury, CT) sonicating water bath with temperature control. About 2 to 5 mg of cleaned glass beads (glyceryl-controlled pore glass particle

DOI: 10.1309/VLA7TT9EQ05FFVN4

size 120-200 mesh; nominal diameter, 350 Å) and 100 µL of the sample preparation buffer (50 mmol/L of potassium chloride; 10 mmol/L of Tris HCl, pH 8.3; 1.5 mmol/L of magnesium chloride; 0.01% gelatin, 0.5% polysorbate 20; and 0.5 mg/mL of Proteinase K) were added to each sample tube. The tubes were then placed in a sonicating water bath at 45°C for 10 minutes.

DNA Purification

Phenol Chloroform.—For the phenol chloroform method,[23] 400 µL of Tris-EDTA saturated phenol (pH 8.0) was added to the sample tube. After centrifugation (12,000 rpm for 10 minutes), the aqueous layer was removed. Next, 400 µL of chloroform/isoamyl alcohol, 24:1, was added to the sample tube, mixed by vortex, and centrifuged (12,000 rpm for 10 minutes). Following removal of the aqueous layer, 1/10 volume of sodium acetate, pH 5.2, and 3 volumes of ice cold, 100% ethanol was added to the solution and kept for 1 hour at –80°C. The sample was then centrifuged at 5°C for 20 minutes. The supernatant was removed, and the pellet was rinsed with 75% ethanol. The sample was resuspended in 25 µL of sterile water and mixed gently in a thermomixer at 37°C for 2 hours.

DNAzol.—For this method,[24] the sample was homogenized in 1 mL of DNAzol reagent (Invitrogen, Carlsbad, CA) and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred to a new tube, and 0.5 mL of 100% ethanol was added and kept for 1 hour at 80°C to precipitate the DNA. The supernatant was removed, and the pellet washed with 75% ethanol. The air-dried pellet was resuspended with 50 µL of Tris-EDTA buffer.

InstaGene.—For the InstaGene method,[25] a volume of InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) was added directly to the sample extraction tube, mixed briefly, and incubated at 56°C for 30 minutes and at 100°C for 8 minutes. The sample was mixed for 10 to 15 seconds and centrifuged at 12,000 rpm for 3 minutes. The supernatant containing isolated DNA was removed.

Boiling.—For the boiling method,[26] the sample was boiled for 8 minutes at 94°C to inactivate the Proteinase K that was added during the digestion method and centrifuged (12,000 rpm for 5 minutes), and the supernatant was transferred to a new tube.

Commercially Available Kits.—DNA extraction was performed according to the manufacturer's instructions using the following kits: (1) QIAamp DNA Mini Kit (Qiagen, Valencia, CA), (2) Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN), (3) Ex-Wax DNA Extraction Kit (Chemicon, Temecula, CA), (4) Nucleon HT Genomic DNA Extraction Kit (Amersham Biosciences, Piscataway, NJ), and (5) MagneSil Genomic, Fixed Tissue System (Promega, Madison, WI).

Following DNA isolation using the various protocols, the quality of the extraction was evaluated by PCR amplification of 4 amplicons ranging in size from 199 to 300 bp.

Polymerase Chain Reaction

PCR amplifications were performed in a final volume of 25 µL containing the following: 2 µL of DNA isolated from FF-PET; 20 pmol of each primer; 200 µmol/L of each deoxyribonucleoside triphosphate; 50 mmol/L of potassium chloride; 10 mmol/L of Tris HCl, pH 8.3; 2.0 to 2.5 mmol/L of magnesium chloride; and 2.0 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Thermocycling conditions were as follows: initial denaturation at 94°C for 10 minutes; 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. To assess the quality of PCR amplification, 8 µL of PCR reaction material was analyzed by agarose gel electrophoresis.

RYR2 Mutational Analysis by DHPLC and DNA Sequencing

Mutational analysis of 25 of the CPVT1-associated exons (30 PCR amplicons) (8, 14, 15, 44-47, 49, 83, 87-95, 97, and 100-105) in *RYR2* by DHPLC and DNA sequencing was performed on DNA derived from FF-PET for 35 unrelated cases of SUD as described previously.[27-29] For samples identified with a nonsynonymous variant (ie, missense mutation), conformational analysis involving another independent DNA extraction from the original FF-PET block, PCR amplification, DHPLC, and sequencing analysis were performed.

Statistical Analysis

Results are expressed as mean ± SD. We used 1-way analysis of variance and the Student *t* test for unpaired data to compare the yields for the amplification reaction. To examine the differences of successful DNA deparaffinization and extractions, the 2-tailed Fisher exact test was used.

Results

Step 1: Initial Evaluation of DNA Isolation Protocols

Three FF-PET blocks were processed using 26 permutations involving 2 deparaffinization methods, 2 standard protocols proposed for digestion, 4 purification methods, and 5 commercial kits. The quality of the DNA extraction was evaluated by PCR amplification of 4 amplicons ranging in size from 199 to 300 bp. Data from this analysis are summarized in **Figure 1A**. Overall, there was no difference in deparaffinization methods in terms of efficacy in the extraction and amplification of DNA (xylene, 62/156 [39.7%] compared with microwave, 68/156 [43.6%]; $P = .56$). However, when using microwave deparaffinization, overnight digestion with Proteinase K was

DOI: 10.1309/VLA7TT9EQ05FFVN4

superior to sonification (25/48 [52%] vs 10/48 [21%], respectively; $P < .003$), whereas no difference was found when xylene was used for deparaffinization (13/48 for Proteinase K vs 15/48 for sonification; $P = .82$).

Although there was significant variability among the 8 standard laboratory-based methods, the commercial kits provided a greater overall yield of analyzable DNA (77/120 [64.2%] vs 63/192 [32.8%] for the laboratory-based methods; $P = .001$). Among the 5 commercially available kits and the 8 laboratory-based methods, 6 protocols (Ex-Wax, MagneSil Genomic, Puregene, QIAamp, Proteinase K–overnight DNAzol, and Proteinase K–overnight InstaGene) gave the best yield of PCR amplification (8/12 amplicons); therefore, these 6 protocols were further analyzed with an increased number of FF-PET blocks in step 2.

Step 2: Evaluation of the Best Protocols Derived in Step 1

By using the 6 best extraction methods (4 commercial kits and 2 laboratory-based methods) obtained in step 1, the number of FF-PET blocks analyzed was extended to 11. For this analysis, both deparaffinization procedures (xylene and microwave) were compared for all 6 extraction methods, and data are summarized in **Figure 1B**. The MagneSil Genomic kit provided a significantly greater yield of analyzable DNA than did the Ex-Wax kit (55/88 [63%] vs 41/88 [47%], respectively; $P = .03$), whereas when the MagneSil Genomic was compared with the other methods, no significant difference was found (Puregene, 46/88 [52%]; Proteinase K–overnight DNAzol, 46/88 [52%]; Proteinase K–overnight InstaGene, 43/88 [49%]; and QIAamp DNA Mini Kit, 43/88 [49%]; $P = .76$). However, the MagneSil Genomic kit was considered

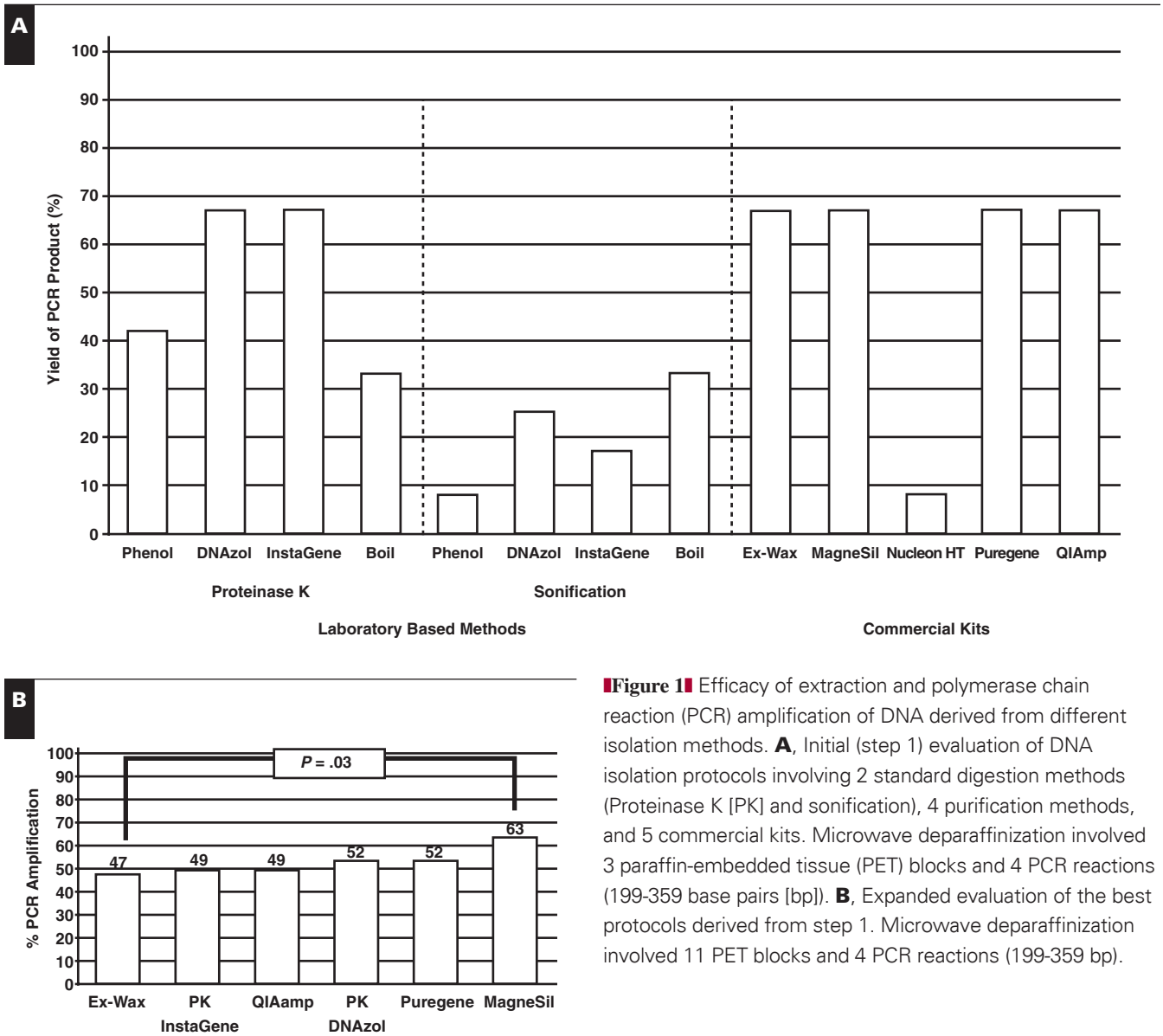

Figure 1 Efficacy of extraction and polymerase chain reaction (PCR) amplification of DNA derived from different isolation methods. **A**, Initial (step 1) evaluation of DNA isolation protocols involving 2 standard digestion methods (Proteinase K [PK] and sonification), 4 purification methods, and 5 commercial kits. Microwave deparaffinization involved 3 paraffin-embedded tissue (PET) blocks and 4 PCR reactions (199-359 base pairs [bp]). **B**, Expanded evaluation of the best protocols derived from step 1. Microwave deparaffinization involved 11 PET blocks and 4 PCR reactions (199-359 bp).

DOI: 10.1309/VLA7TT9EQ05FFVN4

the best in terms of efficacy in extraction and amplification. The microwave deparaffinization results were comparable or slightly superior to those of the xylene method (140/264 [53.0%] vs 134/264 [50.8%]; $P = .66$). Because it requires less time, fewer numbers of manipulations, and is nontoxic, as compared with xylene, microwave deparaffinization was considered the preferred method.

To validate the ability to identify gene mutations using FF-PET–derived DNA, an FF-PET sample from a person previously shown to harbor a *KCNQ1* mutation using autopsy material from blood was extracted using our preferred method (microwave deparaffinization followed by MagneSil Genomic kit DNA isolation) and subsequently analyzed for the known mutation using DHPLC and DNA sequencing. Heteroduplex analysis by DHPLC showed an abnormal profile for this sample, and DNA sequencing confirmed the exact *KCNQ1* mutation that was identified previously.

Feasibility of FF-PET–Derived DNA for Postmortem Genetic Testing

To determine the feasibility of FF-PET–derived DNA for postmortem genetic testing, mutational analysis by DHPLC and DNA sequencing involving 30 PCR amplicons representing 25 of the CPVT1-associated exons of the *RYR2* gene was performed on DNA derived from 35 unrelated cases of SUD. Overall, 11 (31%) of 35 DNA samples could be analyzed fully for the 25 *RYR2* exons, 20 (57%) were analyzed partially, and 4 (11%) could not be amplified. For the 30 amplicons tested, 23 ± 7 amplicons could be PCR amplified and analyzed from FF-PET–derived DNA samples (N = 35). The ability to extract the DNA and to achieve positive PCR amplification was not influenced by the age of the FF-PET block. However, the length of the PCR amplicon significantly influenced the number of positive PCR amplifications derived from the 35 SUD samples, from 27 ± 2 for the 7 amplicons shorter than 230 bp to 21 ± 5 for the 7 amplicons longer than 290 bp ($P = .006$).

Following mutational analysis by DHPLC and direct DNA sequencing, 18 unique DNA alterations—4 intronic polymorphisms, 9 synonymous (silent) variants, and 5 nonsynonymous (missense) variants—were observed among the 35 SUD cases, some representing known common polymorphisms (ie, 464-8 C>A, S453S). **Figure 2** depicts the abnormal DHPLC profiles (panel a) and sequence chromatograms (panel b) for 2 of the 5 novel nonsynonymous *RYR2* mutations (*H469Y, L2299F, A3909T, A4070T,* and *K4481R*) identified in 5 (14%) of 35 cases of SUD. These putative pathogenic *RYR2* mutations were absent in 400 reference alleles (100 healthy white and 100 healthy black volunteers), conserved across species, and localized to key functional domains in the *RYR2*-encoded calcium release channel. Sequence analysis performed in the reverse direction confirmed the presence of the nucleotide change. However, following repeated DNA isolation from the original FF-PET block and mutational analysis, we could not confirm these mutations in any of the 5 cases (Figure 2, panel c), despite confirming common polymorphisms originally seen in these cases, suggesting that these DNA alterations were in fact FF-PET–derived "DNA artifacts" introduced somewhere during the process of tissue preparation, fixation, and paraffinization; the DNA extraction process; or PCR amplification rather than representing legitimate mutations.

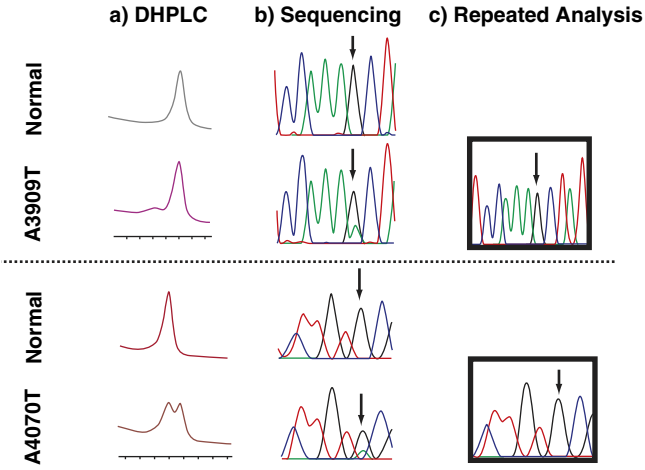

Figure 2 Postmortem mutational analysis from formalin-fixed, paraffin-embedded tissue (FF-PET)–derived DNA and subsequent identification of FF-PET–derived artifacts on repeated analysis. Depicted are the abnormal denaturing high-performance liquid chromatography (DHPLC) profiles (a) and sequence chromatograms (b) for 2 novel heterozygote nonsynonymous *RYR2* mutations (A3909T and A4070T) detected in cases of SUD. Illustrated is the repeated sequence analysis (c) starting from a new DNA extraction for each case. The arrows point to the nucleotide alteration in question for each case.

Discussion

Genomic DNA isolated from archived FF-PET potentially has important applicability in determining the cause and manner of conventional autopsy–negative SUD on a case-by-case basis. In addition, it may serve as an extremely valuable resource in large, retrospective genetic epidemiologic studies on SUD, including sudden infant death syndrome, in which most often FF-PET is the only source for DNA.

The amount of intact DNA that can be extracted successfully from an archival FF-PET block depends on variables usually beyond the control of an investigator, such as the preservative used in tissue processing, the length of fixation, and the age of the specimen.[21] Different tissues show

DOI: 10.1309/VLA7TT9EQ05FFVN4

different yields of DNA. However, successful PCR proved more related to the extraction protocol and formalin fixation than to individual tissues and the quantity of material.[30]

We compared different deparaffinization, extraction, and purification methods to define the best protocol to obtain DNA from FF-PET that is qualitatively suitable to proceed with postmortem genetic testing of one of the most common genetic causes of conventional autopsy–negative SUD, namely CPVT1.[30] By comparing standard laboratory methods and commercial kits, we demonstrated that the extraction method with the greatest yield of analyzable DNA was the commercially available MagneSil Genomic, Fixed Tissue System. In addition, the suitability for mutation analysis was confirmed by investigating FF-PET isolated from a case of SUD with a known mutation in exon 9 of *KCNQ1* (LQT1) that was identified previously by using autopsy material from blood.

But more important, we assessed the feasibility for "whole gene" comprehensive postmortem mutational analysis by extracting DNA from 35 SUD cases using the best methods and subjecting these samples to molecular analysis for 25 of the *RYR2* exons implicated in CPVT1. Although we were able to obtain high-quality DNA from the majority of samples, only one third of the decedents had comprehensive interrogation of all 25 exons. In fact, even with the "best" method, nearly one third of the regions of interest could not be examined on average. Of note, the amplification was not influenced by the age of the blocks. However, because we do not know the time elapsing between autopsy and tissue sampling, we cannot exclude that a different interval could be a cause of the analytic failure for some samples. Moreover, the success of PCR was strongly dependent on the size of the amplified product. There was a significant failure rate for amplicons exceeding 290 nucleotides.

By DHPLC and DNA sequence analysis, different common and rare (novel) single nucleotide polymorphisms, including 5 resulting in putative pathogenic *RYR2* missense mutations, were identified among our 35 cases of SUD, suggesting that nearly 15% of this SUD cohort may have died suddenly due to a fatal, CPVT-triggered ventricular arrhythmia. At first glance, this observation would seem to confirm our initial 15% frequency of authentic *RYR2* mutations in conventional autopsy–negative SUD.[30] However, although the common single nucleotide polymorphisms were confirmed by repeated analysis starting from a new DNA extraction, we could not confirm any of the novel *RYR2* mutations, suggesting that these represented false-positive PCR artifacts. It is important to note that the artifacts occurred during or before the PCR reaction and were not related to the sequencing procedure as suggested by the presence initially of the DNA alteration in the forward and reverse sequence chromatograms.

Artificial mutations resulting from PCR amplification using DNA derived from FF-PET have been described previously with alternative explanations given, including postmortem deamination of cytosine residues resulting in the conversion of cytosine to uracil in the DNA,[31] damaged DNA resulting in strand breaks that promote "jumping" between templates during PCR,[32] and the use of low copy numbers of DNA templates in the PCR reaction.[33]

Considering that conventional autopsy–negative SUD accounts for a significant number of sudden deaths in young people and that epidemiologic, clinical, and now postmortem genetic analyses all attest that approximately one third of SUD cases after the first year of life stem from an inherited lethal cardiac channelopathy,[34] the cardiac channel molecular autopsy should be viewed as the standard of care for the postmortem evaluation of SUD. Thus, postmortem genetic testing, which provides an answer 35% of the time and may lead to saving a surviving family member's life, should become readily available, and the role of the medical examiner, coroner, and/or forensic pathologist is vital because current standard operating procedures for the conduct of an autopsy do not ensure that a postmortem sample is acquired in a DNA-friendly manner. With rare exceptions, FF-PET samples constitute suboptimal sources for postmortem genetic testing for SUD. In contrast, blood collected in EDTA (purple top tube) or frozen heart, liver, or spleen provide the greatest source of intact DNA, permitting the successful conduct of postmortem cardiac channel genetic testing.[15]

It is of extreme importance that guidelines central to the procurement of DNA-friendly sources be added to the standard of care for the postmortem analysis of SUD to ensure an accurate diagnosis and enable informed genetic counseling for families that may guide the appropriate commencement of strategies targeted toward the prevention of another tragedy among survivors.[6]

From the Departments of [1]Medico-diagnostic Sciences and Special Therapies, University of Padua Medical School, Padua, Italy; and [2]Medicine, Pediatrics, and Molecular Pharmacology & Experimental Therapeutics; Mayo Clinic, Rochester, MN.

Supported by a Mayo Foundation Clinical Research Award (Rochester), Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Program (Rochester), the Dr Scholl Foundation (Northbrook, IL), the CJ Foundation for SIDS (Hackensack, NJ), the Hannah Wernke Memorial Foundation (Strongsville, OH), an Established Investigator Award from the American Heart Association (Dallas, TX), and grant HD42569 from the National Institutes of Health, Bethesda, MD (Dr Ackerman) and a grant of the Registry of Cardio-cerebro-vascular diseases, Veneto Region, Venice, Italy (Dr Carturan).

Address reprint requests to Dr Ackerman: Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory, 200 First St SW, Guggenheim 501, Mayo Clinic College of Medicine, Rochester, MN 55905.

DOI: 10.1309/VLA7TT9EQ05FFVN4

Dr Ackerman is a consultant for Clinical Data, which provides the commercially available, clinical genetic test for LQTS called FAMILION. No support was provided for the conduct of this study.

** Dr Carturan was a visiting fellow.*

References

1. Chugh SS, Kelly KL, Titus JL. Sudden cardiac death with apparently normal heart. *Circulation.* 2000;102:649-654.
2. Maron BJ, Shirani J, Poliac LC, et al. Sudden death in young competitive athletes: clinical, demographic, and pathological profiles. *JAMA.* 1996;276:199-204.
3. Liberthson RR. Sudden death from cardiac causes in children and young adults. *N Engl J Med.* 1996;334:1039-1044.
4. Corrado D, Basso C, Thiene G. Sudden cardiac death in young people with apparently normal heart. *Cardiovasc Res.* 2001;50:399-408.
5. Thiene G, Corrado D, Basso C. Cardiomyopathies: is it time for a molecular classification? *Eur Heart J.* 2004;25:1772-1775.
6. Behr E, Wood DA, Wright M, et al. Cardiological assessment of first-degree relatives in sudden arrhythmic death syndrome. *Lancet.* 2003;362:1457-1459.
7. Morentin B, Suarez-Mier MP, Aguilera B. Sudden unexplained death among persons 1-35 years old. *Forensic Sci Int.* 2003;135:213-217.
8. Puranik R, Chow CK, Duflou JA, et al. Sudden death in the young. *Heart Rhythm.* 2005;2:1277-1282.
9. Wever EF, Robles de Medina EO. Sudden death in patients without structural heart disease. *J Am Coll Cardiol.* 2004;43:1137-1144.
10. Ackerman MJ. Cardiac channelopathies: it's in the genes. *Nat Med.* 2004;10:463-464.
11. Ackerman MJ. Cardiac causes of sudden unexpected death in children and their relationship to seizures and syncope: genetic testing for cardiac electropathies. *Semin Pediatr Neurol.* 2005;12:52-58.
12. Goldenberg I, Moss AJ, Zareba W. Sudden cardiac death without structural heart disease: update on the long QT and Brugada syndromes. *Curr Cardiol Rep.* 2005;7:349-356.
13. Ackerman MJ, Tester DJ, Porter CJ, et al. Molecular diagnosis of the inherited long-QT syndrome in a woman who died after near-drowning. *N Engl J Med.* 1999;341:1121-1125.
14. Priori SG, Napolitano C, Giordano U, et al. Brugada syndrome and sudden cardiac death in children [letter]. *Lancet.* 2000;355:808-809.
15. Ackerman MJ, Tester DJ, Driscoll DJ. Molecular autopsy of sudden unexplained death in the young. *Am J Forensic Med Pathol.* 2001;22:105-111.
16. Ackerman MJ, Siu BL, Sturner WQ, et al. Postmortem molecular analysis of SCN5A defects in sudden infant death syndrome. *JAMA.* 2001;286:2264-2269.
17. Chugh SS, Senashova O, Watts A, et al. Postmortem molecular screening in unexplained sudden death. *J Am Coll Cardiol.* 2004;43:1625-1629.
18. d'Amati G, Bagattin A, Bauce B, et al. Juvenile sudden death in a family with polymorphic ventricular arrhythmias caused by a novel *RyR2* gene mutation: evidence of specific morphological substrates. *Hum Pathol.* 2005;36:761-767.
19. Basso C, Calabrese F, Corrado D, et al. Postmortem diagnosis in sudden cardiac death victims: macroscopic, microscopic and molecular findings. *Cardiovasc Res.* 2001;50:290-300.
20. Banerjee SK, Makdisi WF, Weston AP, et al. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. *Biotechniques.* 1995;18:768-770.
21. Greer CE, Wheeler CM, Manos MM. Sample preparation and PCR amplification from paraffin-embedded tissues. *PCR Methods Appl.* 1994;3:S113-S122.
22. Heller MJ, Burgart LJ, TenEyck CJ, et al. An efficient method for the extraction of DNA from formalin-fixed, paraffin-embedded tissue by sonication. *Biotechniques.* 1991;11:372-374.
23. Ghossein RA, Ross DG, Salomon RN, et al. Rapid detection and species identification of mycobacteria in paraffin-embedded tissues by polymerase chain reaction. *Diagn Mol Pathol.* 1992;1:185-191.
24. Taback B, Hashimoto K, Kuo CT, et al. Molecular lymphatic mapping of the sentinel lymph node. *Am J Pathol.* 2002;161:1153-1161.
25. Kleymenova E, Walker CL. Determination of loss of heterozygosity in frozen and paraffin embedded tumors by denaturating high-performance liquid chromatography (DHPLC). *J Biochem Biophys Methods.* 2001;47:83-90.
26. Shibata DK, Arnheim N, Martin WJ. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J Exp Med.* 1988;167:225-230.
27. Tester DJ, Kopplin LJ, Will ML, et al. Spectrum and prevalence of cardiac ryanodine receptor (RyR2) mutations in a cohort of unrelated patients referred explicitly for long QT syndrome genetic testing. *Heart Rhythm.* 2005;2:1099-1105.
28. Tester DJ, Spoon DB, Valdivia HH, et al. Targeted mutational analysis of the RyR2-encoded cardiac ryanodine receptor in sudden unexplained death: a molecular autopsy of 49 medical examiner/coroner's cases. *Mayo Clin Proc.* 2004;79:1380-1384.
29. Ackerman MJ, Tester DJ, Jones G, et al. Ethnic differences in cardiac potassium channel variants: implications for genetic susceptibility to sudden cardiac death and genetic testing for congenital long QT syndrome. *Mayo Clin Proc.* 2003;78:1479-1487.
30. Legrand B, Mazancourt P, Durigon M, et al. DNA genotyping of unbuffered formalin fixed paraffin embedded tissues. *Forensic Sci Int.* 2002;125:205-211.
31. Hofreiter M, Jaenicke V, Serre D, et al. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Res.* 2001;29:4793-4799.
32. Williams C, Ponten F, Moberg C, et al. A high frequency of sequence alterations is due to formalin fixation of archival specimens. *Am J Pathol.* 1999;155:1467-1471.
33. Akbari M, Hansen MD, Halgunset J, et al. Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner. *J Mol Diagn.* 2005;7:36-39.
34. Tester DJ, Ackerman MJ. The role of molecular autopsy in unexplained sudden cardiac death. *Curr Opin Cardiol.* 2006;21:166-172.

DOI: 10.1309/VLA7TT9EQ05FFVN4