Journal of Forensic Research
ISSN: 2157-7145

Piniewska et al., J Forensic Res 2012, 3:10
DOI: 10.4172/2157-7145.1000175

Research Article Open Access

The Comparison of Paraffin Dewaxing Using Methyl Tert-Butyl Ether and Xylene in DNA Extraction from Autopsy Specimens

Danuta Piniewska[1], Marta Wojtas[1], Nina Polańska[1], Agnieszka Stawowiak[1], Marta Konieczna-Waśkowska[1], Magdalena Czepiec[1] and Marek Sanak[2]*

[1]*Department of Forensic Medicine, Jagiellonian University Medical College, Krakow, Poland*
[2]*Department of Medicine, Jagiellonian University Medical College, Krakow, Poland*

Abstract

Formalin fixed and paraffin-embedded tissue specimens collected during surgery or autopsy, are an important source for retrospective diagnosis and identification purposes. Genomic DNA degradation or PCR amplification inhibition are the major cause of DNA amplification failure. Routinely, xylene is used to remove paraffin from paraffin-embedded tissue sections. We tested methyl tert-butyl ether (MTBE), as an alternate organic solvent, which is less harmful for organism than xylene.

Using different tissues (heart, kidney, liver) from randomly selected autopsies (n=10) we compared performance of MTBE and xylene for removal of paraffin during a preparation process compatible with automated staining equipment. All these extracted DNA samples were amplified and genotyped using human identification Identifiler multiplex.

Our experiments points that there is no difference in the range of genotyped microsatellite loci, regardless MTBE or xylene dewaxing. The heart specimen has the highest number of successfully genotyped STR loci, followed by the kidney and the liver. For the genomic template above 260 base pairs of the length no products were obtain from the routinely collected autopsy material. It is worthy of note that MTBE is safer than xylene and according to the current European Community regulations no chemical fume hoods are required for MTBE handling. Thus, MTBE might be preferred to remove paraffin from tissue specimens in forensic or histopathology laboratories not equipped with systems of airborne exposure protection.

Keywords: Paraffin-embedded tissues; Methyl tert-butyl ether (MTBE); Xylene; Paraffin removal; STR genotyping

Introduction

Formalin fixed and paraffin-embedded tissue specimens are an important source of archival DNA for retrospective molecular diagnosis or identification purposes in forensic medicine. Quite often this is the only biological material stored for years in hospital pathology units [1,2]. There are numerous issues with the extraction of high-quality DNA from this type of biological samples, influenced by time and quality of formaldehyde fixation. Usually, nucleic acids are fragmented due to formation of cross-link bonds between DNA and nuclear proteins [3,4]. However, initial step of paraffin removal and tissue rehydration seems pivotal for the yield of DNA, independently to subsequent steps of extraction [5]. Furthermore, genetic material isolated from this source of tissue may contain inhibitors that negatively affect PCR amplification [6-8]. Among other factors determining the quality of genomic DNA preserved in histopathological samples, are interval between removal of tissue and the fixation period, both having a negative impact if prolonged. Alternative fixation reagents, e.g. glutaraldehyde [9] are not popular because of being irritant more than formaldehyde and commonly causing occupational sensitization or asthma. Routine usage of carbonate buffered formalin and precautions to avoid cross-contamination between samples are advocated to obtain tissue samples manageable for DNA studies [10].

Xylene is the routinely used organic solvent to remove paraffin from paraffin-embedded tissue. Chemically xylene is a mixture of three *ortho*-, *meta*- and *para*-dimethylbenzene isomers. This is a volatile liquid, which expose laboratory personnel for intoxication via inhalation, ingestion, and direct eye or skin contact. In the organism, xylene distributes and accumulates in fat, adrenal gland, marrow, spleen and nervous tissue. Exposure to high levels of xylene vapor causes irritation of conjunctiva, nose and throat, headaches. Severe intoxication can lead to symptoms of irritability, nausea and vomiting, with subsequent motor and balance disturbances. There are casuistic reports on depression and aplastic anemia or leukopenia elicited by a chronic occupational exposure to xylene. At high concentrations xylene can cause cardiac dysrhythmia, loss of consciousness [11]. The current European Union directive on hazardous substances (67/548/EEC) classified xylene as harmful (Xn). Animal models for xylene inhalatory toxicity estimate lethal dose 50% (LD50) at the concentration of 5000 ppm of xylene during 4 hours (LD50) [11].

The purpose of this study was to evaluate an alternate organic solvent for removal paraffin from paraffin-embedded tissues. We tested methyl tert-butyl ether (MTBE) and compared its performance to xylene. MTBE is classified as irritant substance. The maximum permissible concentration of MTBE in a work area is allowed 300 mg/m^3, threefold greater than xylene (100 mg/m^3). Toxicology testing

***Corresponding author:** Dr. Marek Sanak, Department of Medicine, Jagiellonian, University Medical College, Skawinska 8, 31-066 Krakow, Poland, Tel: +48 12430 53 72; Fax: +48 12430 52 03; E-mail: nfsanak@cyf-kr.edu.pl

Received December 14, 2012; **Accepted** January 23, 2013; **Published** January 25, 2013

Citation: Piniewska D, Wojtas M, Polańska N, Stawowiak A, Konieczna-Waśkowska M, et al. (2012) The Comparison of Paraffin Dewaxing Using Methyl Tert-Butyl Ether and Xylene in DNA Extraction from Autopsy Specimens. J Forensic Res 3:175. doi:10.4172/2157-7145.1000175

for methyl tert-butyl ether in rabbits evidenced irritation of the eye conjunctiva and no irritation of the skin. A rat test estimate of LD50 for MTBE is 23.576 g/m^3, which is at least fourfold greater than xylene [12]. The Biohazard characteristics of both substances are summarized in Table 1. A suggestion that MTBE is less hazardous than xylene prompted us to validate MTBE as a solvent suitable for dewaxing and not affecting the quality of genotyping results.

Material and Methods

The routine human histopathological autopsy specimens were randomly selected from the repository in the Department of Forensic Medicine, Jagiellonian University Medical College. Representing ten autopsies (7 males), each samples set comprised three types of tissues: heart, liver, and kidney. No post-mortem putrefactive changes were observed in these specimens. Tissues were preserved in 10% carbonate buffered formalin for 2 -5 days. Paraffin-embedded tissues were stored at room temperature in the darkness. Before DNA isolation, tissues samples were cut using a rotary microtome into 8 μm thin sections and attached onto microscope slides. Each processed tissue sample combined two microtome sections. This thickness of microtome cut was found optimal on the basis of previous experiments, and sections did not detached during subsequent baths. Duplicate tissue samples were processed in parallel with either solvent tested for removal of paraffin. Reagents were xylene (POCH, Gliwice, Poland) or methyl tert-butyl ether (MTBE, Sigma-Aldrich, Poland). During the procedure, **microscope slides** were submerged two times for 15 minutes into a staining jar filled with the organic solvent at room temperature. Afterwards, the solvent was removed by two washing in dehydrated ethanol for 5 minutes, next the slides were rinsed with distilled water for 15 seconds and left to dry up at the ambient temperature. Using a sterile cotton swab moistened with 50 ml of deionized water, tissue was collected from the slides and transferred to Eppendorf tubes by cutting off the end of the swab. Genomic DNA was isolated using the enzymatic method (Sherlock AX Kit, A&A Biotechnology, Poland). This kit has a purification step using ion exchange adsorptive column and precipitation the eluent. Dried precipitate was dissolved in 30 μL of deionized water. DNA solution was used as a template for PCR amplification in 2 μL volume. Genotyping of autosomal microsatellite loci was done using AmpFISTR Identifiler Kit (Applied Biosystems, USA) with conditions recommended by the manufacturer and 30 amplification cycles. Multiplex PCR products were separated by electrophoresis in 5,25% denaturing polyacrylamide gel (Amresco, USA) using a G5 compensation matrix and internal size standard Gene Scan-500 LIZ (Applied Biosystems, USA). Electrophoresis gel image was analyzed using GeneScan Analysis 3.7 NT software. Allelic labels of the amplification products were assigned using ABI Prism Genotyper 3.7 NT program by comparison with the allelic ladder included with AmpFISTR Identifiler Kit. Samples processing were done by a technician blinded to the organic solvent used. The correctly ascertained genotype was assumed if either heterozygous, or in a case of homozygosity, the same result had to be present in any other samples of the same individual. Thus, allelic dropout was detected in a tissue sample if only one allele was present, but another tissue or solvent test revealed heterozygosity. Comparisons of numbers of successfully and partially genotyped loci between tissues and solvents were performed using contingency tables. Paired tissue types were compared using Wilcoxon test. A non-parametric Spearman correlation was calculated between number of correctly typed individual samples and the upper bound of PCR product size of the locus. Type I statistical error less than 0.05 was assumed as significant.

Results

The average area of human tissues sample used for DNA extraction was 169 mm^2 ± 8 mm^2 (range from 102 to 213 mm^2). There was no difference in the amount of the material processed for DNA extraction between the three tissues types (heart vs. kidney vs. liver: 2.85 ± 0.46 vs. 2.67 ± 0.44 vs. 2.61 ± 0.52 mg). Quality of genomic DNA template isolated from paraffin-embedded tissues was estimated by percentage of successfully ascertained genotypes of 10 subjects using 15 analyzed microsatellite loci and amelogenin sex marker of Identifiler multiplex (Table 2). The average of successfully typed markers depended on the tissue source used for DNA extraction. The heart muscle specimen had overall 6.38 ± 4.14 loci per subject success rate for xylene and 5.13 ± 4.22 loci per subject for MTBE. For the kidney specimen the success rate was 4.38 ± 4.5 vs. 4.75 ± 3.84 and for liver specimen 3.88 ± 3.44 vs. 3.88 ± 3.30 of loci per subject. No difference between xylene vs. MTBE extraction was detected. The genotyping failure was present in more than 50% analyzed markers. Allelic dropout was noted in 15 MTBE and 7 xylene processed templates using altogether 480 genetic marker amplifications. Failure rate was significantly correlated with the type of tissue and length of the amplification product. For the heart specimen Spearman non-parametric correlation between number of correctly typed individual samples with the highest size of locus was R=-0.91 for xylene and R=-0.89 for MTBE. No PCR products were obtained in case of amplification products exceeding 260 base pairs. Thus, both a small concentration and degradation of genomic DNA was present in these samples. To test for the template efficiency of extracted DNA, amelogenin X locus was selected because amplification products were present in each instance. The area under the peak of X chromosome was compared for all subjects' samples studied using paired Student's t-test. There were no differences in the amplification signal for DNA processed with xylene or MTBE (xylene vs. MTBE: 19538 ± 11874 vs. 17812 ± 12050; p=0.2).

Discussion

Paraffin embedded histopathological specimens are routinely prepared following surgery or autopsy and stored for a long period of time. Thus, this is invaluable source of DNA for molecular studies including mutational screening, oncological diagnostics, retrospective molecular epidemiology of infectious diseases or reference material in forensic investigations [2,13-15]. Quality of the genomic DNA depends on several factors like type of a tissue specimen, time between sampling and fixation of a tissue, chemical reagents used and timing of the consecutive steps of histological processing. To a much lesser extent it

Reagent	Xylene	Methyl tertiary-butyl ether
Hazardous substance symbol	harmful (Xn)	irritant (Xi)
Maximum permissible concentration	100 mg/m^3	300 mg/m^3
Maximum permissible momentary concentration	350 mg/m^3	not established
Topical exposure in rabbits	intense irritation both eyes and skin	slight irritation of the eyes and no irritation of the skin
Inhalatory systemic exposure in rats (lethal concentration, 50%)	5 g/m^3	23.57 g/m^3

Table 1: Safety characteristics of the used paraffin solvents: xylene and methyl tertiary-butyl ether.

	Heart		Kidney		Liver		Product size range (base pairs)
	Xylene	MTBE	Xylene	MTBE	xylene	MTBE	
amelogenin	10/10	10/10	10/10	10/10	9/10	9/10	107 (X), 113 (Y)
D8S1179	10/10	10/10	9/10	9/10	8/10	8/10	123-170
D19S433	10/10	10/10	6/10	8/10	6/10	6/10	102-135
D3S1358	10/10	10/10	6/10	7/10	6/10	6/10	112-140
vWA	9/10	10/10	9/10	9/10	8/10	8/10	155-207
D21S11	9/10	9/10	6/10	7/10	6/10	6/10	185-239
D5S818	9/10	9/10	4/10	6/10	6/10	6/10	134-172
TPOX	9/10	8/10	7/10	8/10	5/10	5/10	222-250
TH01	9/10	7/10	6/10	7/10	6/10	4/10	163-202
D16S539	7/10	6/10	3/10	2/10	2/10	3/10	252-292
FGA	7/10	5/10	3/10	2/10	0/10	1/10	215-355
D13S317	2/10	3/10	1/10	1/10	0/10	0/10	217-245
D18S51	1/10	2/10	0/10	0/10	0/10	0/10	262-345
D7S820	0/10	1/10	0/10	0/10	0/10	0/10	255-291
D2S1338	0/10	0/10	0/10	0/10	0/10	0/10	307-359
CSF1PO	0/10	0/10	0/10	0/10	0/10	0/10	305-342

Table 2: Numbers of correctly typed loci in three different tissues types from 10 human autopsies, and for xylene or Methyl Tertiary-butyl Ether (MTBE) removal of paraffin.

depends on the time of storage of the paraffin embedded blocks or glass slide-mounted sections of a tissue. The most critical step to maintain DNA integrity is fixation. Formaldehyde solution (10%) buffered to the neutral pH is routinely recommended. Fixation time should not be longer than 72 hours to prevent crosslinking of DNA with nuclear proteins or nucleotide deamination [9,10]. Removal of paraffin is regarded as a preparatory step before DNA extraction; however, its completeness is mandatory for optimal yield of the nucleic acids extraction. This step also contributes in removal of common inhibitors of PCR amplification like a complex tissue hydrolysate containing heme, hemosiderin, acid polysaccharides and lipids [6,7]. Xylene is the routinely used organic solvent verified to thoroughly remove paraffin. However, because of its toxicity attempts were made to subside xylene with another dewaxing method [16] during a classical histopathologic preparation. The aim of our study was to compare efficacy of xylene with MTBE. Estimated toxicity of MTBE is at least 3 times lower than that of xylene. Using a typical human tissue material collected during 10 autopsies, we did not find any difference between these solvents. Moreover, we reconfirmed that number of correctly genotyped loci is limited by a degradation of genomic DNA, which hampers PCR amplification of templates longer than 260 bp [8]. Incomplete results of genotyping can be improved by the use of a higher amount of the tissue for extraction, but it cannot prevent a difference between signal strength of smaller versus larger amplicons [17,18]. Extent of DNA degradation is variable in different tissues and depends on the time between the death and fixation of the collected samples. In general, histological samples from the biopsy procedures give much better results of genotyping [19].

We observed significant differences in the number of correctly typed loci, between different tissues, sampled during the same autopsy. It could be explained by the water content and presence of enzymes, lipids and polysaccharides by which DNA gets decomposed post mortem. Among three tissue types we tested, the best results were obtained for the heart muscle, next was the kidney and the worst the liver. These tissues correspond to the most frequently collected during autopsy. Our results are in agreement with other publications on the quality of genomic DNA from the autopsy samples [9,10]. In our comparison of xylene versus MTBE, testing three different tissues in parallel was required also to detect allelic dropout. This source of genotyping error turned out moderately frequent and was noticeable in 1.5% xylene and 3.1% MTBE extracted tissue sections.

The proposed method of tissue section processing is compliant with automated staining instrumentation because is done on microtome sections adherent to glass slides. Only the last step requires a manual collection of the hydrated section using a standard swab, from which DNA is next extracted using the same procedure as for buccal swabs. We deliberately did our experiments on a limited tissue sections volume. Assuming the average surface of tissue sample of two paraffin sections, 8 μm of thickness each, DNA extraction was completed from 2.7 ± 0.12 mg (±standard deviation) of rehydrated biologic material. This, at the best corresponds to 1-2 ng of DNA in 30 μL of the extract. Thus, DNA amplification were possible form as low amount as 0.2 ng of the genomic template. No measurements of DNA concentration were done for two reasons. The commercial reagents kit, we use for extraction, has the final purification step, which uses co-precipitation of DNA with blue-dextran. This interferes with fluorimetric measurements of DNA concentration. The other method, based on the quantitative real-time amplification of human repetitive sequences does not reflect the template quality of a degraded genomic material. Therefore, we assumed that serial microtome sections contain similar amount of tissue, which seemed a reasonable assumption to test for solvents used for paraffin removal.

The main advantage of the alternate organic solvent-MTBE is its lower toxicity, which at least formally, do not require active air-exchange environment. MTBE can be recommended as a xylene substitute for removal of paraffin. According to the presented method, compatible with automated processing of glass slides, it is expected that at least 6 microtome sections of 8 μm of thickness would be sufficient to obtain genomic DNA required for successful amplification and genotyping using human identification Identifiler multiplex. Extraction of DNA from the heart or kidney tissue and design of mini-STR or short amplicons reactions can enhance the chance for complete genotyping using paraffin embed autopsy samples.

Acknowledgement

Supported by the grant from the Polish Ministry of Science and Higher Education for Leading National Research Centers (KNOW).

References

1. Shibata D, Kurosu M, Noguchi TT (1991) Fixed human tissues: a resource for the identification of individuals. J Forensic Sci 36: 1204-1212.

2. Romero RL, Juston AC, Ballantyne J, Henry BE (1997) The applicability of

formalin-fixed and formalin fixed paraffin embedded tissues in forensic DNA analysis. J Forensic Sci 42: 708-714.

3. Feldman MY (1973) Reactions of nucleic acids and nucleoproteins with formaldehyde. Prog Nucleic Acid Res Mol Biol 13: 1-49.
4. Lehmann U, Kreipe H (2001) Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods 25: 409-418.
5. Greer CE, Lund JK, Manos MM (1991) PCR amplification from paraffin-embedded tissues: recommendations on fixatives for long-term storage and prospective studies. PCR Methods Appl 1: 46-50.
6. Cao W, Hashibe M, Rao JY, Morgenstern H, Zhang ZF (2003) Comparison of methods for DNA extraction from paraffin-embedded tissues and buccal cells. Cancer Detect Prev 27: 397-404.
7. Coura R, Prolla JC, Meurer L, Ashton-Prolla P (2005) An alternative protocol for DNA extraction from formalin fixed and paraffin wax embedded tissue. J Clin Pathol 58: 894-895.
8. Bonin S, Petrera F, Niccolini B, Stanta G (2003) PCR analysis in archival postmortem tissues. Mol Pathol 56: 184-186.
9. Miething F, Hering S, Hanschke B, Dressler J (2006) Effect of fixation to the degradation of nuclear and mitochondrial DNA in different tissues. J Histochem Cytochem 54: 371-374.
10. Legrand B, Mazancourt Pd, Durigon M, Khalifat V, Crainic K (2002) DNA genotyping of unbuffered formalin fixed paraffin embedded tissues. Forensic Sci Int 125: 205-211.
11. Chempur (2009) Material Safety Data Sheet for xylene.
12. Poch (2008) Material Safety Data Sheet for methyl tert-butyl ether.
13. Carturan E, Tester DJ, Brost BC, Basso C, Thiene G, et al. (2008) 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. Am J Clin Pathol 129: 391-397.
14. Crisan D, Mattson JC (1993) Retrospective DNA analysis using fixed tissue specimens. DNA and Cell Biol 12: 455-464.
15. Banaschak S, Du Chesne A, Brinkmann B (2000) Multiple interchanging of tissue samples in cases of breast cancer. Forensic Sci Int 113: 3-7.
16. Falkeholm L, Grant CA, Magnusson A, Möller E (2001) Xylene-free method for histological preparation: a multicentre evaluation. Lab Invest 81: 1213-1221.
17. Farrugia A, Keyser C, Ludes B (2010) Efficiency evaluation of a DNA extraction and purification protocol on archival formalin-fixed and paraffin-embedded tissue. Forensic Sci Int 194: e25-e28.
18. Coates PJ, d'Ardenne AJ, Khan G, Kangro HO, Slavin G (1991) Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. J Clin Pathol 44: 115-118.
19. Chen X, Shen YW, Gu YJ (2005) The research of relationship between DNA degradation and postmortem interval. Fa Yi Xue Za Zhi 21: 115-117.