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The novel genomic DNA integrity in neutral buffer formalin, Bouin's fluid, and zinc formalin fixatives: A comparative study

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Abstract

DNA extraction was successfully performed on all samples with variable concentrations. Fifteen liver samples were subjected to the extraction technique, with five liver samples utilized from each fixative, this comparison study aimed to assess DNA integrity across different fixatives. DNA was successfully extracted from all samples regardless of their varying quantities. Each sample yielded at least one motif with a length of 100 base pairs. All 15 samples produced 100 bp amplicon for the IRBP gene. All 15 samples generated a 200 bp amplicon from the NBF, ZF, and BF. The success proportion dropped with growing amplicon length, by which 66.6% (10/15) of 300 bp amplicons were successfully produced from NBF and ZF, showing significant differences at $P<0.05$ than BF, while no other amplicon length obtained 0% for 400, 500, and 750 bp respectively, on the other hand, frozen samples worked as a control were able to produce all amplicon length from 100bp to 750bp. Only samples from NBF and ZF constantly yielded more extensive amplicon ranges than BF. BF was unable to keep intact DNA fragments of more than 200 bp. Our study concluded that different fixatives could produce at least 100 bp and 200 bp. However, ZF and NBF are more dependable than BF in producing 300 bp for downstream analysis for further next-generation techniques. Furthermore, NBF and ZF are supreme in producing better-quality DNA fragments than BF.

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Introduction

Formalin fixative and paraffin embedding (FFPE) is an essential procedure for the longstanding conservation of most pathological specimens used in lab settings (1). Experimental and clinical diagnostic library sets frequently rely on (FFPE) since many tissue archives are easy to reach (2,3). Despite multiple attempts to create a more efficient technique, extracting DNA from FFPE tissue is still challenging (4,5). Recent papers praising FFPE tissues as a resource for disease study demonstrate that such archives include a wealth of material suitable for pilot biomarker studies and retrospective molecular biology research. The DNA recovered from FFPE specimens is altered and fragmented, making its use difficult (2). Genomic DNA might be lost through the fixation process (6). It is becoming increasingly crucial to understand the fixatives' impact on the stability and practicality of conserved RNA and DNA (7). Over the years, various fixation techniques and fixative kinds have been proposed and evaluated (7,8). Due to its broad tissue and component preservation capabilities, 10% neutral buffered formalin has become the global fixative of choice (9). Results from molecular biology investigations involving DNA extraction from formalin-fixed tissues have been contradictory (10-12). NBF fixation negatively affects nucleic acids, causing minimal production, destruction, and DNA base changes, leading to substantial artifacts (13). DNA solubility is affected by the use of sections, even with a short treatment period with formalin (14). NBF is the primary fixative used in histopathology laboratories; however, alternative fixatives may be utilized for specific applications. The fixative fluid contains a saturated aqueous

solution of picric acid, acetic acid, and formalin (15). Involving acid fixatives like BF in several territories has restricted using archival tissue for molecular research. Acidic conditions are a primary factor leading to DNA breakdown. RNA extraction from these preserved tissues is also challenging (16). However, DNA extracted by this novel method is appropriate for genetic experiments, implying DNA fragments smaller than 200 bp (1). DNA and RNA may be achieved and studied from Bouin's fixed and paraffin wax-embedded tissues (16). Furthermore, the data indicates that Bouin's fixative causes damage to both DNA and RNA. Nevertheless, conducting molecular investigations on such tissue samples is feasible by lowering the amplification fragment size to under 100 base pairs for RNA and less than 200 base pairs for DNA (16). On the other hand, finding the perfect fixative that can preserve tissue shape, protein integrity, and nucleic acid is crucial for the molecular investigation of tissues and cells (17). Additionally, zinc-based fixatives are affordable, prepared, and highly efficient fixatives that improve the conservation of DNA, RNA, and proteins. It enables better PCR, making it a promising alternative to NBF for modern molecular pathobiology research (17,18).

This study aimed to evaluate the quality of DNA integrity from different fixatives in the context of amplicon sizes to be used in downstream molecular analysis.

Materials and methods

Ethical approve

The University of Mosul College of Veterinary Medicine Administrative Animal Care and Use Council approved experiments on animals (UM.VET.2023.058, January 9, 2024).

Tissues

Paraffin tissue blocks were chosen from the different fixatives (NBF, BF, and ZF) included in this study. The animal house is at the University of Mosul, College of Veterinary Medicine. To reduce other resources of dissimilarity, only liver samples are targeted in DNA extraction. Five samples were selected from each fixative, and 15 were performed in this DNA analysis.

Extraction of DNA from paraffin blocks

DNA recovery was performed using the ADD BIO INC DNA FFPE Tissue Kit from Daejeon, Republic of Korea, following the recommended procedure (19,20). All samples were eluted using 50 ml of ATE buffer and incubated for 5 minutes at room temperature. The number of tissue sections used for extraction is equal to that of all samples, 0.033mg. DNA concentrations were measured using the Qubit device.

PCR analysis

The DNA samples were utilized as templates for PCR amplification, using the primer pairs shown in Table 1 to produce multiple products of varying lengths, namely 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, and 750 bp, for IRBP3 as described by (2). In summary, the samples were amplified using PCR Master Mix (Taq ADDBIO INC Master Mix) by MiniAmpPlus Thermocycler (Applied Biosystems, by Thermo Fisher Scientific with the following conditions: 95ºC for 5 min, followed by 35 cycles of 95ºC for 20 sec, 62ºC for 20sec, then 72ºC for 20 sec. This was followed by 5 min at 72ºC, then held at 12ºC. The DNA was isolated from tissues that were previously stored at a temperature of -20 degrees Celsius, using the ADD BIO INC DNA FFPE Tissue Kit (Daejeon, Republic of Korea) and DNase-free water as positive and negative controls, correspondingly (2,21). Different, forward primers were programmed in different reactions (Uniplex PCR was achieved) in the thermocycler with other conditions to achieve a good result for each set, while the gel electrophoresis worked as all DNA reaction results were run once to have the bands get in order (22).

Table 1: Primer sets (DNA primers utilized in this study)

No.	Primer name	Product size (bp)	Primer Sequence 5'-3'	Length
1.	IRBP-FI	100 bp	GAGCCGATATGCAAGGGTGA	20
2.	IRBP-RI		ATCTTCTGGGATGTGTGCCG	
3.	IRBP-F2	200 bp	CGACATCGAGGGTCACAGAG	20
4.	IRBP- R2		GTAGATGCCCACGGAGAGTG	
5.	IRBP -F3	300 bp	CGACATCGAGGGTCACAGAG	20
6.	IRBP-R3		AGTGATGTCGGGTTCCACAC	
7.	IRBP F4	400 bp	CCT KGTRCTGGANATGGC	18
8.	IRBP R4		CGGAGRTCYAGCACCAAGG	19
9.	IRBP F4	500 bp	CCT KGTRCTGGANATGGC	18
10.	IRBP R5		GATCTCWGTGGTNGTGTTGG	20
11.	IRBP F4	750 bp	CCT KGTRCTGGANATGGC	18
12.	IRBP R6		CTCAGCTTCTGGAGGTCC	

Statistical analysis

Both inferential and descriptive statistics were done with JMP Pro 16.1. Significant frequency differences were found in the findings using chi-square analysis. At P< 0.05, the findings were important. JMP Pro16.1 Software, SAS Institute Inc., Cary, NC, 2021 (23).

Results

Polymerase chain reaction (PCR) enables us to utilize FFPE tissues for further research information to investigate the cause of diseases. DNA was successfully and variably extracted from all samples. Fifteen liver samples participated in extraction procedures, and five samples were used from each fixative for a relative study to evaluate DNA integrity from different fixatives. We could extract DNA from all tissue samples with different concentrations, as shown in Table 2.

Table 2: DNA Concentration (quantities) ng/ML

NBF	ZF	BF	Frozen samples
2.06	4.03	4.46	1.68
69.8	5.77	2.64	>600
5.62	36.0	23.5	>600
13.4	8.2	17.66	>600
40.3	20.47	32.4	>600

A minimum of a single motif was produced from every sample with 100bp. 100% (15/15) of samples had a 100 bp, and 100% (15/15) of 200 amplicons were generated from NBF, ZF, and BF figures 1, 2 and 3. The success proportion decreased with increasing amplicon size, by which only 66.6% (10/15) of 300 bp amplicons were successfully produced from NBF and ZF, while no other amplicon length obtained 0% for 400 bp, 500 bp, and 750 bp respectively, on the other hand, frozen samples worked as a control produced all amplicon lengths from 100 bp to 750 bp. Only samples from formalin and zinc formalin consistently produced the larger amplicon sizes contrasted to BF figures 1and 2. The Bruins fluid could not keep intact DNA fragments of more than 200 bp.

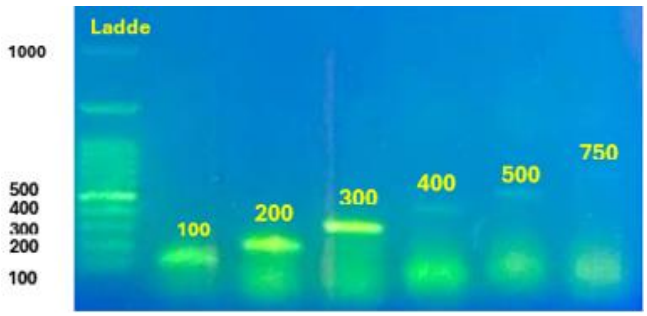

Figure 1: Bands of frozen samples.

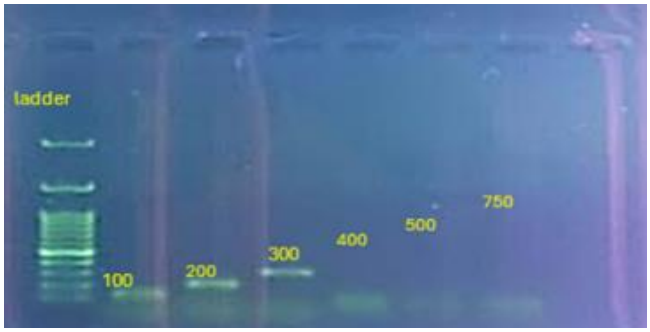

Figure 2: Bands of NBF samples.

The type of fixative chemical structures substantially impacted DNA fragment length, as substantial samples were produced from tissues sharing similar chemical structures compared to the remaining samples. All but five samples from Bouin's fluid generated 100 and 200 bp amplicons; however, both NBF and ZF samples exceeded to amplify the 300bp but failed for the long fragment, such as 400 bp and beyond, from the IRBP3 gene. Yet the fresh samples successfully produced all amplicon sizes and 500 bp and 750 bp. Although formaldehyde degrades DNA, causing significant destruction and often producing small fragments in most FFPE samples, it was found in our study that 66.6% of the samples were still able to amplify fragments that were 300 base pairs long and 100% of 100 and 200 bp (Figures 1-4).

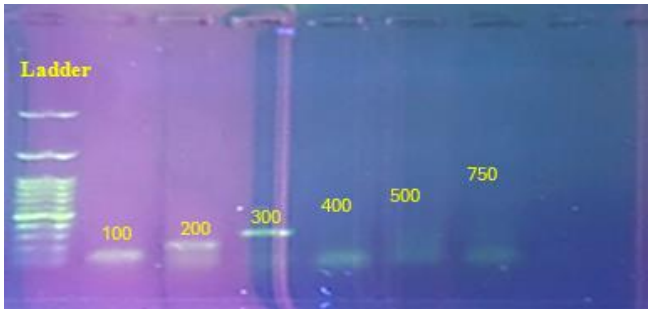

Figure 3: Bands of ZF samples.

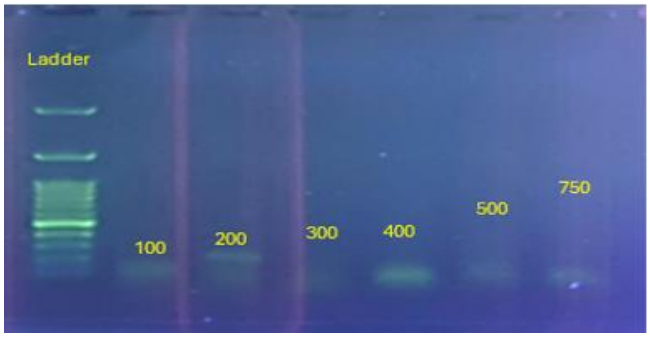

Figure 4: Bands of BF samples.

Discussion

Each fixation method was determined to be a suitable DNA resource for molecular investigations, and they facilitated the effective PCR amplification of a specific target gene (24). FFPE samples are highly valuable for identifying biomarkers in data analysis and eventual biomarker detection (25). Today, FFPE samples are the ever-increasing advanced research technique concerning investigating biological agents and treating tumors (26), making them more valuable than ever. The polymerase chain reaction (PCR) enables the use of these tissues for further research of disease pathophysiology. Although formalin degradation of DNA can cause considerable damage and often result in modest product sizes in most necropsy samples, all fixatives included in this investigation were able to amplify fragments that were 200 base pairs long. While much data indicates that formaldehyde causes DNA destruction, only a limited number of research have shown the production of large-sized DNA from tissues preserved in formalin (27), which agrees with what we had of amplicon length from BF in this study. Different methods for DNA extractions are now experiencing amplification failure when utilizing these approaches with archival fixed tissues (28), which is compatible with the DNA concentration variances we were able to measure in our study. These 'failures' can occur not just because the target DNA sequences are missing in the tissues but also because of the specific fixative that was utilized. The choice of nucleic acid extraction method will also impact the amplification outcomes (28). As we mentioned previously, BF failed to amplify long DNA fragments in contrast to NBF and BF, even though their ability to produce large amplicons was limited to 300bp. This result matches previous studies such as Baloglu *et al.* (24), which mentions that most DNA molecules found were smaller than 100 bp in size. Our data regarding the product size of samples fixed in BF was inadequate as we could not gain more than 200 fragment lengths, and this result is related to the acidic nature of Bouin's fixative, which causes damage to both DNA and RNA (16). Nevertheless, the amplification process may yield positive results when the length of the amplicon is decreased to around 100-200 base pairs for DNA. Which fully agreed with our findings (16).

Conclusions

It frequently retrieves and examines DNA from Bouin's preserved and paraffin-embedded tissues from BF. Our study concluded that Neutral Buffer Formalin, Bouins Fluid, and Zinc Formalin could produce at least 100 bp and 200 bp. However, ZF and NBF are more dependable than BF in producing 300 bp for downstream analysis for further next-generation techniques. Furthermore, NBF and ZF are superior in producing better quality DNA fragments than BF, which successfully produced 100 and 200 base pairs.

Conflict of interest

The writers of this article have not revealed any potential conflicts of interest.

Acknowledgment

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سلامة الحمض النووي الجينومي في مثبتات الفورمالين الدارئ المتعادل، وسائل بوين، فورمالين الزنك: دراسة مقارنة

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الخلاصة

تم ستخرج الحمض النووي بنجاح من جميع العينات. استخدمت ١٥ عينة من الكبد بواقع خمس عينات من كل مثبت. هدفت الدراسة لمقارنة سلامة الحمض النووي لمثبتات مختلفة وأنتجت كل عينة تسلسل ثابت من القواعد النيتروجينية بطول ١٠٠ قاعدة من البادئ حيث أنتجت جميع العينات ١٠٠ قاعدة نيتروجينية من التسلسل الجيني لبروتين ربط الريتينويد بين المستقبلات الضوئية، أنتجت جميع العينات ٢٠٠ قاعدة نيتروجينية من العينات في جميع المثبتات (محلول الدارئ والزنك فورمالين ومحلول باونس). بينما انخفضت نسبة النجاح مع طول التسلسل الجيني, حيث تم إنتاج ٦٦,٦٪ (١٥/١٠) بطول تسلسلي من القواعد النيتروجينية المحددة بـ ٣٠٠ قاعدة بنجاح من كل من الدارئ المتعادل والزنك فورمالين مما يظهر اختلافات كبيرة عند مستوى معنوية (أ<٠,٠٥) بالمقارنة مع محلول باونس ومن ناحية أخرى كانت النسبة ٠٪ للأطوال المصممة مع البادئ من طول التسلسل الجيني الى ٤٠٠، ٥٠٠ و٧٥٠ أما العينات المجمدة المستخدمة كعينات سيطرة فقد أعطت نتائج مبهرة من خلال القدرة على إنتاج جميع التسلسلات للأطوال المختلفة من التسلسل الجيني من ١٠٠ الى ٧٥٠ قاعدة نيتروجينية. أما محلول باونس فقد تعذر في الحفاظ على أجزاء الحمض النووي سليمة لأكثر من ٢٠٠ من القواعد النيتروجينية. خلصت دراستنا إلى أن المثبتات المختلفة يمكن أن تنتج ما لا يقل عن ١٠٠ و٢٠٠ تسلسل سليم، إضافة الى ذلك فقد تميز كل من محلول الدارئ والزنك فورمالين في الحفاظ على تسلسل سليم وصلت الى ٣٠٠ قاعدة نيتروجينية. فان الاطوال السليمة من التسلسل الجيني يستفاد منها للدراسات الجينية. إن المحلول الدارئ والزنك فورمالين يتفوقان في إنتاج أجزاء من الحمض النووي ذات جودة أفضل من محلول باونس.