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Evaluation of DNA Isolation and Amplification from Various Organs Preserved through Frozen, Formalin-Fixed and Paraffin-Embedded Tissue Sample method

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ABSTRACT

The purpose of this study was to compare the purity, concentration, and DNA band visualization of the isolated sample and PCR amplicon from three sample storage methods i.e. fresh frozen sample (-20^{o}C to -196^{o}C), preserved in formalin, and paraffin wax. For this tissue samples were collected from the sample stored at frozen temperature -20^{o}C, 10% NS formalin, and paraffin-embedded preparations, and Abs260/230 and Abs260/280 values and electrophoresis of 0.8% and 2% agarose gel visualization were analyzed. The results of the study showed a significant value of Abs260/280 for the isolated and amplified DNA purity. Among the tested three methods, frozen sample isolates and the PCR amplicon visualized a good DNA band. Meanwhile, the formalin-fixed and paraffinized tissue storage method showed a slightly lower quality DNA and no DNA band, respectively, while the PCR amplicon visualized a thin DNA band. In conclusion, all the tissue storage methods can be applied for DNA preservation and isolation, and the samples are successfully amplified on PCR examination.

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1 Introduction

The polymerase chain reaction is a molecular technique that allows in vitro DNA and gene amplification (Ehtisham et al. 2016). PCR can be used for diagnostic purposes to detect the presence of a specific DNA sequence of an organism such as an animal, plant, or microbial, it is also used for testing, diagnostics, or varietal selection. These days various types of PCR such as real-time PCR, quantitative PCR, Nested PCR, etc. are available for diagnostic and amplification purposes (Kadri 2019). The isolation of the DNA sample needs to be considered to obtain a good amplified result. This could be achieved by using the appropriate sample storage method which can protect the cell structures and DNA from damage.

The frozen storage method is widely used for tissue preservation at freezing temperatures (-20^{o}C to -196^{o}C) in liquid nitrogen. As a drawback, in this preservation method, there might be a chance of cell inactivation through tissue freezing, thereby inhibiting the rate of DNA damage (Wilson and Walker 2010). Cold temperatures also affect the fluidity, osmotic pressure, and tissue pH Intracellular ice formation can affect the cell's viability while the extracellular ice formation can cause an osmotic shift, leading to a concentration enhancement or reduction of extracellular fluid. This process can also damage cells' either by shrinking or swelling (Seawright et al. 2013). Therefore another alternative method is needed to avoid these reactions and simplify the storage process that does not require too many tools.

Formalin or formaldehyde is also widely used in tissue preservation for morphology, histological, and immunohistochemistry study. The diffusion process assisted formalin penetration into the cells by methylene glycol. In this method, aldehyde dehydrogenase that presents in the cell reacts to formalin and induces protein and nucleic acid cross-linking (Thavarajah et al. 2012; Groelz et al. 2013). Further, protein-DNA crosslinks binding occurs between cysteine, histidine, tryptophan, and lysine to deoxynucleosides, deoxyadenosine, deoxycytidine, and deoxyguanosine. The most common crosslinks are lysine and deoxyguanosine and cysteine and deoxyguanosine (Yu et al. 2015). In this manner, the formalin preservation method becomes potential and simple method of tissue storage for DNA isolation and PCR without using many tools as required in the freezing process.

Paraffin-embedded tissue preservation method has been also used in many clinical pathology laboratories. This method can be used for long-term storage from 1 month up to 5 years, at room temperature. According to Yi (2020), the paraffin-embedded tissue preservation method did not show any significant difference in extracted DNA and RNA purity as compared to the other method. Further, small-sized extracted DNA fragments were successfully amplified from most paraffin block samples for several years at room temperature (Nam et al. 2014). In this manner, this also becomes a potential preservation method for DNA isolation and the PCR amplification method used in medical diagnosis identification.

The pectoralis muscle is a skeletal muscle group that is composed of such muscle fibers that vary in size, shape, and arrangement of protein components. Differences in the number of DNA, RNA, and protein components will reflect cell population, size, and metabolic activity. Further, the chicken pectoralis muscle has a rapid growth and proliferation rate and is characterized by high cellular activities of satellite cells'. The higher tissue's cell proliferation indicates the high amount of DNA and high demand for cellular energy which is facilitated by mitochondria that have their own genetic material mtDNA (Mawaryana 2015).

The liver is one of the metabolic organs in the body and play important role in carbohydrate, lipid, and protein metabolism by bile juice secretion. Due to the high DNA synthesis peak of hepatocytes, the liver has a rapid and abundant regenerative capacity (Yagi et al. 2020). This indicates that the liver has a good amount of DNA to be isolated and amplified.

The chicken Growth Hormone (cGH) gene consists of 191 amino acids, located on chromosome 27 with a 4.1 kb length (Jafari et al. 2015). The cGH gene has a substantial influence on growth and metabolic function and is also expressed as Growth Hormone (GH) for body composition, egg production, reproduction, sexual maturation, and the central nervous system (CNS) functions (Khaerunnisa et al. 2017). This study aimed to compare the purity, concentration, and DNA band visualization of the isolated DNA sample and PCR amplicon from various organs preserved by three sample storage methods i.e. fresh frozen sample (-20^{o}C to -196^{o}C), preserved in formalin, and paraffin wax.

2 Materials and Methods

2.1 Paraffin-embedded preparation

The chicken pectoralis muscle and liver tissue were freshly collected and stored in 10% formalin. For this, 0.5 cm of the concerned tissue was trimmed and inserted into a tissue cassette. The tissue cassette was gradually soaked in ethanol 70%, 80%, 85%, 90%, 95%, ethanol absolute I, II, and III for 1 hour. Then it was soaked in xylol I, II, and III for 10 minutes and paraffin solution I, II, and III for 1 hour, respectively. Finally, the tissue was embedded with paraffin and stored.

2.2 Sample preparation

Broiler chicken liver and pectoralis muscle were collected from 10 Cobb Broiler chickens. In total, 30 samples were used, and these were divided into three equal groups for different storage methods. Five liver and pectoralis muscle tissue were stored at frozen

temperature -20°C, 10% NS formalin, and paraffin blocks. The frozen samples thawed to a lower temperature, and 10% NS formalin samples were rinsed with physiological saline (NaCl 0.9%) 3 times to remove the remaining formaldehyde in the tissue through the pipetting method.

The paraffin block samples were cut 100 µm using a microtome and placed into 1.5 ml PCR tubes. First, paraffin removal of the sample was carried out using 800 µl xylene, and the sample was homogenized with a vortex at a low speed for 10 minutes, then centrifuged at 14.000 rpm for 3 minutes. The supernatant was discarded using a micropipette without disrupting the pellet. This paraffin removal was repeated three times. After 2-3 times deparaffinization, the samples were gradually rehydrated with different alcohol concentrations. First, 800 µl of 100% alcohol was added to the tubes and homogenized with a vortex at a low speed for 10-15 seconds, then centrifuged at 14.000 rpm for 3 minutes. The supernatant was discarded using a micropipette without disrupting the pellet. Subsequently, this method was repeated two times with 800 µl of 70% and 50 % alcohol, and the supernatant was discarded using a micropipette without disrupting the pellet.

2.3 DNA Extraction

The DNA isolation procedure used the standard DNA isolation *Thermo Scientific Gene JET Genomic DNA Purification Kit*. The tissue sample was weighed (20 mg) and placed into a 1.5 ml Eppendorf tube, respectively. A total of 180 µl of digestion solution and 20 µl of Proteinase K were added into the tube and homogenized with a vortex for 10-15 seconds. Then the sample was incubated 2-3 hours at 56°C and homogenized using a vortex for every 30 minutes. After this, 20 µl of RNAse was added and homogenized using a vortex and incubated at room temperature for 10 minutes. This was followed by the addition of 200 µl of lysis solution and homogenization and at the end of the procedure, 500 µl of 50% ethanol was added and homogenized.

The sample was inserted into a collection tube and centrifuged at 6000 g speed for 1 minute. Afterward, the sample was taken in a new collection tube and 500 µl of wash buffer I was added and centrifuged at 8000 g for 1 minute. This was followed by the addition of 500 µl of wash buffer II and centrifuged at maximum speed for 3 minutes. In the end, 100 µl elution buffers were added to the fresh sample tube and 50 µl to the formalin and paraffin-embedded sample tube and incubated at room temperature for 2 minutes. The sample was centrifuged at 8000 g for 1 minute and isolated DNA was collected.

2.4 Polymerase Chain Reaction (PCR)

PCR tubes were prepared and added 2.5 µl of each isolated DNA sample. This was followed by the addition of a 5 µl PCR master mix solution and 2.5 µl Nuclease Free Water (NFW). Each tube was added with 1 µl cGH forward primer (5'-TCCCAGGCTGCGTTTTGTTACTC-3 ') and 1 µl cGH reverse primer (5'-ACGGGGGTGAGCCAGGACTG-3') with 429 bp targeted gene. Afterward, the PCR process was run using *miniPCR® mini8 thermal cycler bio™*.

2.5 Agarose gel electrophoresis

The agarose gel used was 0.8% for the DNA isolation sample and 2% for the PCR amplicon. The 0.8% agarose gel was made with 20 ml TBE (Tris-Borat EDTA) added with 0.16-gram agarose powder in a glass beaker, and 2% agarose gel was made with 20 ml TBE (Tris-Borat EDTA) was added with 0.4 gram agarose powder in another beaker glass. Both mixtures dissolved in a heated stirrer for 10-20 minutes. Subsequently, 2 µl peq-green (*ethidium bromide*) was added to the suspension and then inserted into the electrophoresis tray. It was set at room temperature for 10-15 minutes until the gel was dense.

An Agarose gel tray was placed on the electrophoresis machine. The DNA marker 100 bp DNA ladder, Load Ready™ was added (5 µl) to the first well. Then 10 µl of isolate DNA were added from the second well to the next well respectively, then 2 µl of loading dye (*bromophenol blue/* ethidium bromide) was added to each sample well. Afterward, the gel was run for about 25 minutes in blueGel™ Electrophoresis with a built-in transilluminator.

2.6 Statistical analysis

The purity dan concentration result was analyzed quantitatively using SPSS for windows with a one-way ANOVA statistical analysis. If there was a significant difference, proceed with the Tukey test $\alpha = 0.05$. Meanwhile, the quality was analyzed by the electrophoresis DNA band visualization.

3 Results

Most of the isolated samples have 260/280 nm and 260/230 nm values below the normal average (1.8-2 and 2.0-2.2, respectively). There is a significant value for the Abs 260/280 resulting from all the tissue storage comparisons with 0.000 values (Table 1). The paraffin-embedded pectoralis sample showed the lowest Abs260/230 nm value with 0.85±0.16. Meanwhile, the paraffin-embedded liver sample showed a better value (1.53±0.15). In addition to the Abs260/280 nm value, the paraffin-embedded pectoralis sample also has the lowest value with 0.74±0.18, followed by formalin-fixed pectoralis and liver tissue samples showed a lower value of 260/280nm (0.90 ±0.18 and 0.95 ±0.96 respectively). Paraffin-embedded pectoralis also showed the lowest concentration compared to the other samples, followed by paraffin-embedded liver samples. The Abs260/280 values then proceed to

Tukey test α = 0.05 (Table 3). PCR results showed all storage methods' showed uniform purity and concentration (Table 2). Although all of the purity levels were below the ideal absorbance average value (1.8-2 and 2.0-2.2). A significant value for the Abs260/280 proceeds to Tukey test α = 0.05 (Table 4).

The DNA bands were visualized only for frozen storage isolated samples with more than 3000 bp, while the formalin-fixed and paraffin-embedded isolated samples showed no DNA band (Figure 1A, 1B & 1C). The amplified DNA samples reached to the total DNA target at 429 bp (Figure 2A, 2B & 2C). The fresh-frozen pectoralis sample 1, 4, and 5 showed no amplified DNA band, and all formalin-fixed amplified samples visualized an amplified DNA band (Figure 2A). Fresh frozen and formalin-fixed liver tissue samples showed an amplified DNA band, with a thin band on samples 8, 9, and 10 (Figure 2B). In contrast, all the paraffin-embedded amplified samples showed very thin to no band on samples 6, 7, and 8 (Figure 2C).

Table 1 Mean of DNA isolation sample purity and concentration

DNA Isolation Sample	Abs260/230	Abs260/280	Concentration (ng/µl)
Frozen pectoralis	1.30 ± 0.61	1.07 ± 0.19	242.83 ± 270.79
Formalin-fixed pectoralis	1.08 ± 0.57	0.90 ± 0.18	223.10 ± 208.98
Paraffin-embedded pectoralis	0.85 ± 0.16	0.74 ± 0.08	7.69 ± 3.45
Frozen liver	1.33 ± 0.10	1.04 ± 0.08	114.90 ± 51.67
Formalin-fixed liver	1.05 ± 0.06	0.95 ± 0.96	150.98 ± 69.07
Paraffin-embedded liver	1.53 ± 0.15	1.18 ± 0.59	30.33 ± 10.33
Significancy value (P)	0.073	0.000	0.081

Values are average of five replicates; mean ± SE, *(P<0.05): significant

Table 2 Mean of DNA purity and concentration

PCR Sample	Abs260/230	Abs260/280	Concentration (ng/µl)
Frozen pectoralis	1.87 ± 0.12	1.62 ± 0.12	846.07 ± 169.80
Formalin-fixed pectoralis	1.79 ± 0.05	1.50 ± 0.03	899.68 ± 48.70
Paraffin-embedded pectoralis	1.85 ± 0.01	1.51 ± 0.01	908.46 ± 44.18
Frozen liver	1.83 ± 0.02	1.49 ± 0.06	891.73 ± 52.74
Formalin-fixed liver	1.80 ± 0.04	1.49 ± 0.04	969.70 ± 65.17
Paraffin-embedded liver	1.83 ± 0.02	1.48 ± 0.03	897.94 ± 26.46
Significancy value (P)	0.292	0.018	0.359

Values are average of five replicates; mean ± SE, *(P<0.05): significant

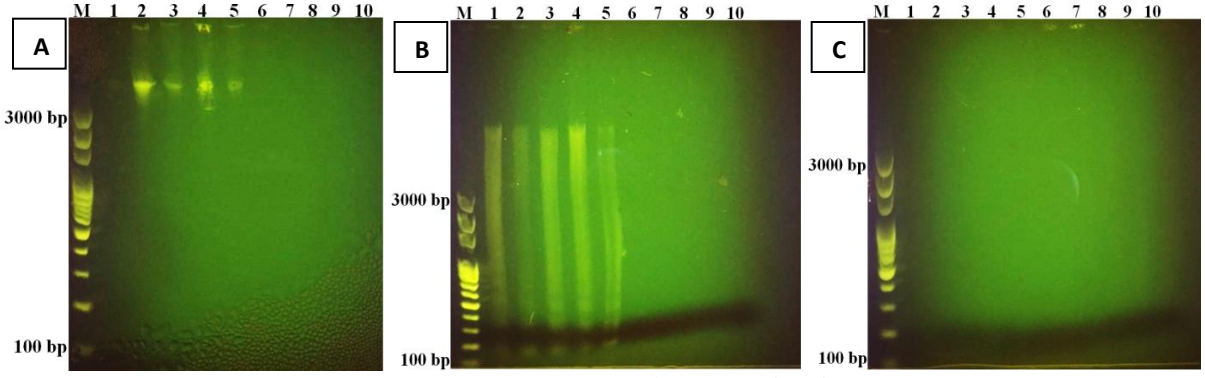

Figure 1 Agarose gel 0.8% concentration showing more than 3000 bp of isolated (A) fresh frozen pectoralis muscle sample storage DNA (1-5), (B) fresh frozen liver sample storage DNA (1-5), and (C) no DNA band of paraffin-embedded pectoralis and liver tissue (Here Lane M: DNA marker 100 bp-3000 bp; lanes 1-5: frozen pectoralis muscle DNA isolate; lanes 6-10: formalin-fixed pectoralis muscle DNA isolate).

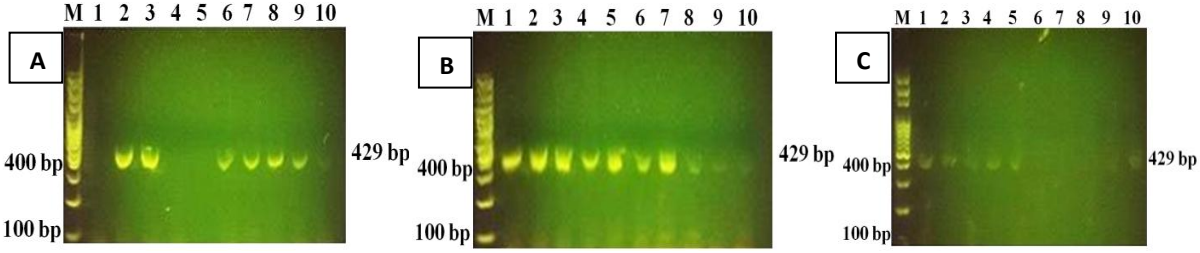

Figure 2 Agarose gel 2% concentration showing PCR amplicon band of (A) fresh frozen and formalin-fixed pectoralis muscle tissue 429 bp, (B) fresh frozen and formalin-fixed liver tissue 429 bp, and (C) paraffin-embedded liver tissue 429 bp (Lane M: DNA marker 100 bp-3000 bp; lanes 1-5: frozen pectoralis muscle PCR amplicon; lanes 6-10: formalin-foxed pectoralis muscle PCR amplicon).

Table 3 Tukey test of the Abs260/280 isolated DNA purity

(I) Storage	(J) Storage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Frozen pectoralis	Formalin-fixed pectoralis	.17200	.07990	.296	-.0750	.4190
	Paraffinated pectoralis	.32800*	.07990	.005	.0810	.5750
	Frozen liver	.02600	.07990	.999	-.2210	.2730
	Formalin-fixed liver	.12000	.07990	.666	-.1270	.3670
	Paraffinated liver	-.11000	.07990	.740	-.3570	.1370
Formalin-fixed pectoralis	Frozen pectoralis	-.17200	.07990	.296	-.4190	.0750
	Paraffinated pectoralis	.15600	.07990	.397	-.0910	.4030
	Frozen liver	-.14600	.07990	.468	-.3930	.1010
	Formalin-fixed liver	-.05200	.07990	.986	-.2990	.1950
	Paraffinated liver	-.28200*	.07990	.019	-.5290	-.0350
Paraffinated pectoralis	Frozen pectoralis	-.32800*	.07990	.005	-.5750	-.0810
	Formalin-fixed pectoralis	-.15600	.07990	.397	-.4030	.0910
	Frozen liver	-.30200*	.07990	.010	-.5490	-.0550
	Formalin-fixed liver	-.20800	.07990	.135	-.4550	.0390
	Paraffinated liver	-.43800*	.07990	.000	-.6850	-.1910
Frozen liver	Frozen pectoralis	-.02600	.07990	.999	-.2730	.2210
	Formalin-fixed pectoralis	.14600	.07990	.468	-.1010	.3930
	Paraffinated pectoralis	.30200*	.07990	.010	.0550	.5490
	Formalin-fixed liver	.09400	.07990	.843	-.1530	.3410
	Paraffinated liver	-.13600	.07990	.544	-.3830	.1110
Formalin-fixed liver	Frozen pectoralis	-.12000	.07990	.666	-.3670	.1270
	Formalin-fixed pectoralis	.05200	.07990	.986	-.1950	.2990
	Paraffinated pectoralis	.20800	.07990	.135	-.0390	.4550
	Frozen liver	-.09400	.07990	.843	-.3410	.1530
	Paraffinated liver	-.23000	.07990	.078	-.4770	.0170
Paraffinated liver	Frozen pectoralis	.11000	.07990	.740	-.1370	.3570
	Formalin-fixed pectoralis	.28200*	.07990	.019	.0350	.5290
	Paraffinated pectoralis	.43800*	.07990	.000	.1910	.6850
	Frozen liver	.13600	.07990	.544	-.1110	.3830
	Formalin-fixed liver	.23000	.07990	.078	-.0170	.4770

*. The mean difference is significant at the 0.05 level; Dependent Variable: Abs260/280; Tukey HSD

Table 4 Tukey test of the Abs260/280 amplified DNA purity

(I) Storage	(J) Storage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Frozen pectoralis	Formalin-fixed pectoralis	.11600	.03923	.067	-.0053	.2373
	Paraffinated pectoralis	.10400	.03923	.123	-.0173	.2253
	Frozen liver	.13000*	.03923	.031	.0087	.2513
	Formalin-fixed liver	.13000*	.03923	.031	.0087	.2513
	Paraffinated pectoralis	.13200*	.03923	.027	.0107	.2533
Formalin-fixed pectoralis	Frozen pectoralis	-.11600	.03923	.067	-.2373	.0053
	Paraffinated pectoralis	-.01200	.03923	1.000	-.1333	.1093
	Frozen liver	.01400	.03923	.999	-.1073	.1353
	Formalin-fixed liver	.01400	.03923	.999	-.1073	.1353
	Paraffinated pectoralis	.01600	.03923	.998	-.1053	.1373
Paraffinated pectoralis	Frozen pectoralis	-.10400	.03923	.123	-.2253	.0173
	Formalin-fixed pectoralis	.01200	.03923	1.000	-.1093	.1333
	Frozen liver	.02600	.03923	.984	-.0953	.1473
	Formalin-fixed liver	.02600	.03923	.984	-.0953	.1473
	Paraffinated pectoralis	.02800	.03923	.978	-.0933	.1493
Frozen liver	Frozen pectoralis	-.13000*	.03923	.031	-.2513	-.0087
	Formalin-fixed pectoralis	-.01400	.03923	.999	-.1353	.1073
	Paraffinated pectoralis	-.02600	.03923	.984	-.1473	.0953
	Formalin-fixed liver	.00000	.03923	1.000	-.1213	.1213
	Paraffinated pectoralis	.00200	.03923	1.000	-.1193	.1233
Formalin-fixed liver	Frozen pectoralis	-.13000*	.03923	.031	-.2513	-.0087
	Formalin-fixed pectoralis	-.01400	.03923	.999	-.1353	.1073
	Paraffinated pectoralis	-.02600	.03923	.984	-.1473	.0953
	Frozen liver	.00000	.03923	1.000	-.1213	.1213
	Paraffinated pectoralis	.00200	.03923	1.000	-.1193	.1233
Paraffinated pectoralis	Frozen pectoralis	-.13200*	.03923	.027	-.2533	-.0107
	Formalin-fixed pectoralis	-.01600	.03923	.998	-.1373	.1053
	Paraffinated pectoralis	-.02800	.03923	.978	-.1493	.0933
	Frozen liver	-.00200	.03923	1.000	-.1233	.1193
	Formalin-fixed liver	-.00200	.03923	1.000	-.1233	.1193

*. The mean difference is significant at the 0.05 level; Dependent Variable: Abs260/280 ; Tukey HSD

4 Discussion

Formalin-fixed tissue samples showed lower DNA isolation purity than the fresh-frozen tissue samples and a higher concentration than the paraffin-embedded tissue sample. A lower purity absorbance is caused by the protein-DNA crosslinks that remain in the isolated sample. The presence of a protein still attached to DNA can lower the purity result. Protein is linked with formalin in the N-terminus site of the amino acid chain, in which the formalin acts as the bridge from amino acids to DNA, where the formalin connects to the base site of DNA. These crosslinks can also induce a higher concentration because formalin protein-DNA crosslinks also induce DNA fragmentation. This fragmentation process potentially gives rise to a higher number of formalin-fixed tissue sample concentrations than the paraffin-embedded ones. This mechanism will alter the hydrogen bonds of nucleic acid base pairs and cause DNA fragmentation. Based on Kennedy-Darling

and Smith (2014), crosslink reversal can be optimally achieved with heat exposure, such as heating the sample at 120^{o}C for 25 to 40 minutes before the DNA isolation process (Mehdi and Reza 2012). Accordingly, one-time temperature exposure in the DNA isolation process is not enough to detach the formed links that affect the DNA purity and concentration.

The paraffin-embedded samples showed the most distinct DNA isolation purity and concentration compared to other storage methods. Long-term paraffin blocks the tissue-making process and potentially causes DNA damage by degradation and denaturation. The tissue soaked in different solutions from dehydration, clearing, and paraffin-embedding processes will influence the cell condition within the tissue. This is related to the cell condition that has to suffer against osmotic to pH changes. Furthermore, a lower purity value can be caused by the paraffin remains in the DNA isolate possibly preventing the aromatic ring of the nitrogenous base from absorbing UV light in the spectrophotometry. Thus, it results in a decrease in absorbance ratio value.

Although fragmentation occurs due to tissue preservation based on the DNA isolation process, the tissue PCR amplicon produced uniform purity and concentration value. Related to the crosslink reversal process, the heating temperature phases in the thermal cycler can be an influencing factor to open the protein-DNA linkage. The repeated heat exposure exponentially helps denature the residual protein-DNA crosslinks that are still attached from the DNA isolation process and become detached entirely so that the DNA is successfully amplified. These results indicate that the DNA fragments from each storage method can still serve as templates for target genes to yield a good value of DNA concentration obtained through amplification.

In electrophoresis visualization, the phosphate group of the negatively charged DNA will be ionized to migrate in the gel matrix toward the positive charge area. The ethidium bromide (EtBr) was added to the samples before electrophoresis to "stain" the DNA. It will bind between the nucleic acid bases that have a hydrophobic region. The hydrophobic environment will induce EtBr to fluorescence by the UV light and visualizing the DNA band. Consequently, the DNA damage will not provide that hydrophobic environment for EtBr to visualize.

Moreover, the DNA damage that already occurs also leads to alteration of ionized DNA structure which will decrease the DNA sensitivity to electrophoresis electric current. Hence, this could be the reason for a good DNA purity and concentration value but results in the absence of a DNA band on the gel visualization. Compared to the fresh frozen samples, the isolated formalin-fixed and paraffin-embedded tissue samples have a good purity ratio but can decrease the DNA quality. Therefore, these samples can still be amplified to the targeted number of genes.

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