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THE EFFECT OF EXTRACTION METHODS , THE KIND OF ORGAN SAMPLES AND THE EXAMINATION DELAY ON THE DNA YIELDS AND TYPING

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INDEXING WORDS

extraction method; human organ; examination delay; DNA typing

SYNOPSIS

This study investigated the effect of DNA extraction methods, examination delay and the kind of organs samples to the DNA yields and typing. Thirty autopsy cases with postmortem period less than 12 hours were used as the sample resources. The DNA was successfully extracted from cerebral cortex, liver, spleen, lymph nodes, kidney, psoas muscle and prostate gland by Bar and Kirby methods. The spectrophotometric measurement showed that the spleen, lymph nodes, kidney and liver provided more DNA rather than the other organs. The agarose gel electrophoresis showed that the majority of the samples had High Molecular Weight-DNA (HMW-DNA) with variable degree of degradation. All of these DNA were successfully typed on the D1S80 locus using the PCR according to Kasai method.

The spleen samples were collected from the same cases and stored at - 20 °C for 1 to 6 weeks before the DNA extraction was

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performed. The analysis of the DNA extracted from these samples showed that the DNA yields and typing did not change significantly among the samples with examination delay up to 6 weeks after the sample collection.

The comparison between the Bar and Kirby methods showed that the Kirby method resulted in more DNA yields with the same purity of DNA, but less HMW-DNA compared with Bar method.

INTRODUCTION

The DNA analysis in the forensic cases has been reported to give the valuable contribution to solve many forensic cases. Comparing with the other forensic samples, such as blood and sperm , the reports on the DNA analysis of the tissue samples are few and sparse 1,2,3). The major problem in the DNA analysis of postmortem tissue is the decomposition that will degrade the DNA 4,5,6,7). Since the presence of High Molecular Weight-DNA (HMW-DNA) is essential for most DNA analysis , especially RFLP (Restriction Fragment Length Polymorphisms) analysis 1,8,9), the major aim of DNA extraction is to get the HMW-DNA as much as possible. The degraded DNA , as far as it contains enough HMW-DNA, can be usually analyzed by various DNA typing methods 10). In the cases with advanced decomposition, the DNA analysis methods are limited 6) and the Polymerase Chain Reaction (PCR) method may be the only method which can be used 5,7,10,). Thus, every effort should be taken to minimize the decomposition by choosing the proper procedure to collect, transport, store and extract the DNA to get the DNA with better quantity and quality.

The different tissue has different DNA content 1,5), depending on the structure and composition of the cells. The tissue which contains more nucleated cells and fewer connective tissue usually provides more DNA. In the case in which many tissues are available, the samples should be taken from the tissue that contains more DNA. This study investigated the DNA yields and typing of the various kinds of organs in order to find the suitable organs for DNA analysis in forensic materials.

Indonesia is a big country with more than 17,000 islands. This condition causes the transportation problem from one place to another. In the Indonesian context, when the cases happen somewhere, the samples must be collected and then be transferred to a few Forensic Centres which can analyze the DNA. In many cases the distance is very far and the examination is delayed for days or even weeks. As far as the facilities for proper storage and transportation are available, there is no problem. However, in the majority of the cases , the only storing method available is to store the samples at 0 or -20 °C. This study investigated the effect of storing the samples at -20 °C for 1 to 6 weeks on the DNA yields and typing.

The DNA extraction methods on the tissue materials are basically the same as other DNA extraction methods. The differences among the methods are usually in the complexity, in the kind of chemicals used and the presence of modification, addition or reduction of the step of conventional procedures. The method of choice on DNA extraction is that which consumes less time and less chemicals but can provide the more amount of DNA , more HMW-DNA, more purity and the capability to be typed. Bar 1) and Kirby 11) methods are two of a few DNA extraction methods for tissue samples. These two methods have big differences in chemical consumption and complexity, however the comparison about the effectiveness of these methods in extracting DNA has not been reported. This study investigated the effect of extracting DNA by Bar and Kirby methods on the DNA yields and typing.

MATERIALS AND METHODS

Materials of this study were 30 autopsy cases with the postmortem period less than 12 hours in the Department of Forensic Medicine, Faculty of Medicine, University of Indonesia, Jakarta and the Department of Legal Medicine, Kobe University School of Medicine. Only the cases without massive bleeding were included in this study.

Influence of the kind of organs.

The tissue specimens were taken from the cerebral cortex, hilar lymph nodes, liver, spleen, kidney, prostate gland and psoas muscle. From every case about 50 grams of tissue were taken, wrapped by aluminum foil and stored at - 20 °C until the DNA extraction was performed within 12 hours after the autopsy. The DNA was extracted either by Bar 1) or by Kirby 11) method. From every organ more than two specimens was taken.

Influence of examination delay.

As much as 20 grams of spleen tissue were taken from the above cases, wrapped using aluminum foil and stored at -20 °C. The DNA was extracted either by Bar or by Kirby method within 12 hours after the sample collection and the extraction was repeated every week up to 6 weeks. The comparison was made among the samples with the examination delay 1 to 6 weeks.

Influence of DNA extraction method.

The DNA extracted by Bar and Kirby methods from the above experiments was analyzed by comparing the DNA yields, the HMW-DNA recovery and the degradation stage of the DNA.

The DNA yields were calculated spectrophotometrically according to the theory that 50 ug/ml of double stranded DNA solution has 1.0 value of optical density at 260 nm. DNA content per 100 mg sample was calculated based on the above result. The purity of DNA was calculated by counting the ratio of OD260/OD280, in which the pure DNA has the ratio 1.80 or more 12). The HMW-DNA and the degradation of the DNA were evaluated by performing electrophoresis on 0.7 % agarose gel with ethidium bromide staining. The lambda/Hind III digest DNA was used as the marker DNA. The HMW-DNA was the uncut-DNA with the molecular weight 23 kb or more 8). The DNA was typed on the locus D1S80 by PCR according to Kasai method 13). The PCR products were analyzed on polyacrylamide gel electrophoresis (PAGE) by ethidium bromide staining. For the statistical analysis the Chi square test was used.

RESULTS

Influence of the kind of organs

All the samples analyzed in this study provided variable amounts of DNA in variable degradation stages. Even among the samples from the same individual the variation of DNA yields was very large. This condition made the standard deviation large. Among the six organs, the spleen, lymph nodes, liver and kidney provided the most abundant DNA (Table I). The electrophoresis pattern of the DNA recovered from postmortem tissue had the smear pattern, quite different from the DNA recovered from blood which usually gives the band pattern (Figure 1). This smear pattern showed that the DNA was partially degraded. The majority of the DNA recovered from spleen, kidney and lymph nodes were HMW-DNA, while the other only showed the DNA of about 10 to 23 kb fragment. All of the DNA were successfully typed on D1S80 locus and gave one or two bands on the gel. The DNA typing was identical among the samples of various organs as far as it derived from the same person (Figure 2).

Influence of examination delay

Examination delay from 1 to 6 weeks seemed to have no effect on the DNA yields and degradation process as far as the sample was stored at - 20 °C. Figure 3 showed that all of the samples with examination delay from 1 to 6 weeks had the same degradation stage. The spectrophotometric analysis of the DNA showed no reduction of DNA yields among the samples from various examination delays (Table II).

Influence of DNA extraction method

The comparison between Bar and Kirby methods showed that the DNA recovery of Kirby method was more than that of Bar method ($X^2 = 44.168$, $p < 0.001$) at the rate of 1.39 times. Macroscopically, the DNA extracted by Kirby method was white, while the DNA extracted by the Bar method very often showed the yellowish to brownish discoloration, although the spectrophotometric assessment of the

TABLE I.

The DNA yields (µg/100 mg) and the purity (OD260/OD280) of various organ samples, extracted by Bar and Kirby methods. The data of lymph nodes is only from Bar method due to lack of materials.

Organ sample	Bar method			Kirby method		
	DNA yields ($\bar{X}$±SD)	Purity	N	DNA yields ($\bar{X}$±SD)	Purity	N
Spleen	267.80 ± 111.60	1.417	58	512.11 ± 101.30	1.525	38
Liver	157.10 ± 36.23	1.628	58	389.30 ± 134.30	1.673	38
Kidney	143.90 ± 65.30	1.685	58	202.40 ± 58.20	1.975	38
Prostate gland	81.27 ± 35.21	1.509	38	159.80 ± 100.90	2.005	30
Psoas muscle	60.79 ± 31.05	1.806	58	88.97 ± 41.92	1.870	38
Cerebral cortex	81.80 ± 43.85	1.744	58	65.80 ± 32.50	1.908	38
Lymph nodes	180.05 ± 49.51	1.555	57	No data		

Statistical analysis : Chi-square

DNA yields : x^2 = 44.168 , $p < 0.001$

Purity : x^2 = 0.0405, not significant.

TABLE II.

The DNA yields (µg/100 mg) and the purity (OD260/OD280) of spleen samples with extraction delay from1 to 6 weeks, extracted by Bar and Kirby methods.

Examination delay(week)	Bar method			Kirby method		
	DNA yields($\bar{X}$±SD)	Purity	N	DNA yields($\bar{X}$±SD)	Purity	N
0	267.80 ± 111.60	1.417	58	512.11 ± 101.30	1.526	38
1	251.70 ± 93.60	1.466	58	416.80 ± 91.80	1.861	36
2	276.80 ± 98.04	1.528	58	338.90 ± 76.65	1.932	36
3	283.20 ± 101.90	1.631	57	354.20 ± 101.30	1.803	34
4	355.80 ± 112.90	1.591	57	327.30 ± 100.50	1.681	34
5	316.20 ± 78.64	1.629	56	368.30 ± 81.26	1.766	32
6	327.50 ± 89.05	1.675	54	347.80 ± 75.80	1.732	28

Statistical analysis : Chi-square

DNA yields : x^2 = 65.767 , $p < 0.001$

Purity : x^2 = 0.0357, non significant

TABLE III.

The comparison between Bar and Kirby methods on the chemicals and time consumption to extract DNA from 500 mg of tissue sample.

Bar method		Kirby method	
Cell digestion :			
1 ml of Tris HCl	10 mM	50 ml of Tris HCl	100 mM
EDTA	10 mM	EDTA	40 mM
SDS	2 %	SDS	0.2 %
		NaCl	100 mM
		DTT	40 mM
Proteinase K	40 µg	Proteinase K	2500 µg
		50 ml of Tris HCl	100 mM
		EDTA	40 mM
Phenol extraction:			
Phenol	1.5 ml	Phenol	125 ml
Methylene Chloride	2.5 ml	CIAA *	75 ml
Dialysis :			
No need		TES solution **	3000 ml
Butanol concentration:			
No need		1-butanol	100 ml
		diethyl ether	50 ml
Ethanol precipitation :			
Sodium acetat 3M	100 ul	Sodium acetate 3M	5 ml
Ethanol	2.5 ml	Ethanol	125 ml
Time consumption :			
3 days		2 days	

* CIAA; chloroform/isoamyl alcohol

** TES ; Tris/EDTA/sodium chloride

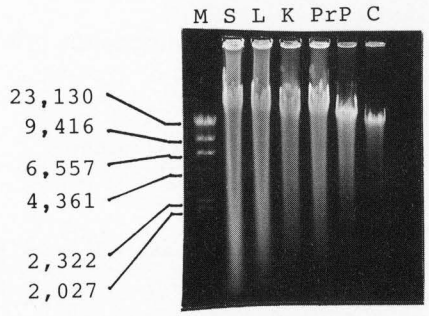

Figure 1.

The DNA extracted from various tissue samples were electrophoresed in 0.7 % agarose, stained with ethidium bromide and visualized by UV light. The lambda/HindIII digest was used as the DNA marker (M). All of the DNA recovered from spleen (S), liver (L), kidney (K), prostate gland (Pr), psoas muscle (P) and cerebral cortex (C) had the HMW-DNA (molecular weight is 23 kb or more). The smear pattern means that these DNA had partially degraded due to decomposition.

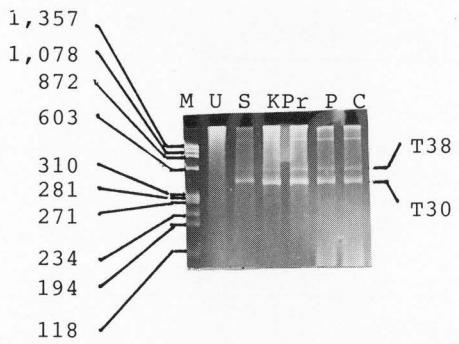

Figure 2.

The PCR-based DNA analysis of the locus D1S80 by Kasai method was performed on the DNA extracted from spleen (S), kidney (K), prostate gland (Pr), psoas muscle (P), cerebral cortex (C). The PCR products were analyzed on polyacrylamide gel electrophoresis (PAGE) and stained by ethidium bromide. The HaeIII digest DNA was used as the DNA marker (M). U was unamplified DNA sample. The typing of these samples were all the same (T30/T38) because all of the samples were taken from the same person.

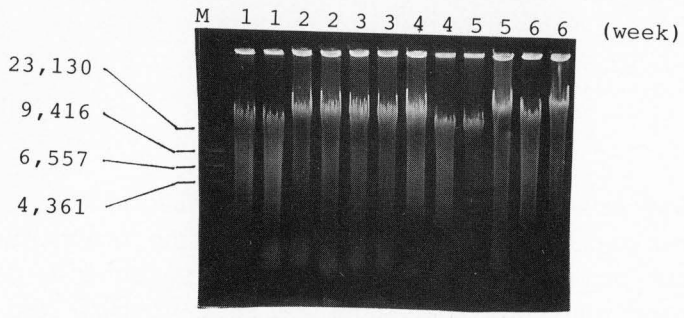

Figure 3.

The spleen samples were taken from the autopsy case and stored at -20 °C. The DNA extraction was performed after storage for 1, 2, 3, 4, 5 and 6 weeks. The analysis of the DNA on 0.7 % agarose gel electrophoresis showed that all of the DNA were in the same condition. It means that there was no progression of degradation during this period. The HindIII digest DNA was used as the DNA marker (M).

purity of the DNA showed no significant difference (X^2 = 0.0405, not significant) (Table I).

The electrophoresis analysis showed that the HMW-DNA recovery by the Bar method was higher than that by Kirby method (610 out of 725 samples or 84.25 % versus 260 out of 420 samples or 61.90 %).

DISCUSSION

In the forensic cases, the samples are often limited in kinds, amount and also quality 5,7,9,14). Since DNA from the postmortem samples is usually degraded , some efforts should be performed to get the DNA with good quality and quantity as well as to minimize the progression of degradation, otherwise DNA analysis will be difficult or even cannot be performed anymore 10,11). The efforts should be made for early collecting samples, choosing the proper samples, proper handling and storing, early extraction and using the proper analysis methods. Since the reports about this topics are sparse, we performed this study, especially for application in Indonesian condition.

In the case of an incomplete body, such as the tissue found in the bumper of a car in a hit and run case, we have no chance to choose the sample and all available sample must be analyzed. However, in the cases with complete body, we can choose the kind of samples we want to analyze 1,15). The DNA contents of every tissue depend on the structure of the organs and the relative amount of the nucleated cells in the tissue. In this study we found that lymphoid organs, such as spleen, lymph nodes contained the most abundant DNA, followed in order by the liver and kidney. While the prostate gland, psoas muscle and cerebral cortex contained fewer DNA than the above organs. These findings confirmed Bar study 1), which examined the organs with postmortem period from 6 to 19 hours. The study by Ludes et al 9) showed that the lymph nodes, kidney, spleen and liver had higher DNA yields than the other organs.

In choosing the proper samples , the stability of the DNA of postmortem samples should be taken into consideration 1,6,9,15,16). As soon as the cells died , the DNA will be degraded by

endonucleases and exonucleases, which belong to the hydrolases 1,4,6). The endonucleases will shear the DNA into smaller fragments, whereas the exonucleases detach from nucleotide one after another from the end terminal, thus gradually shortening the DNA 1). Bar study 1) showed that the DNA fragments gradually disappeared , at first the long fragment DNA (15 - 20 kb). In the agarose gel electrophoresis, the degraded DNA will give the smear pattern 1,4,6,9,10,12,13,15) rather than the band pattern as usually found in the blood or sperm sample (Figure 1). Among the postmortem organs, cerebral cortex 1,9), lung 3) lymph nodes 1,9) , psoas muscle 1,6,9,12,13,16) , heart muscle 9,13,15), spleen 15), kidney 9), bone 5,10,15) and particularly blood 1,12,13) showed the better DNA stability rather than the other organs 1). On the other hand, the liver tended to be more degraded than other organs 1,9,15). Thus, it is unwise to choose the liver in the case of a decomposed body. In the case of a very advanced decomposition and postmortem period of more than 3 weeks, the only available sample is brain 9). The DNA in the samples fixed with buffered formalin and those embedded in paraffin are also well preserved for years 17,18).

If all kinds of samples are available, Kirby suggested to take more than one organ because the DNA quality may be varied among the organs, depending on the action of DNAase 11). In relatively fresh samples, the spleen, lymph nodes, liver and kidney should be chosen because they contain much DNA. However in the decomposed body, the samples of cerebral cortex (whatever the macroscopic appearance), lungs, lymph nodes, kidney and muscle are recommended because they have more stable DNA.

Generally, low temperature 1) and low humidity 1,4) are the best condition for preservation of the DNA samples for a few days without excessive DNA degradation. However, if the examination will be delayed more than 48 hours, they should be frozen at - 20 to - 80 °C. Ogata et al 6) had preserved the muscle at - 25 °C for 10 months and still got the proper DNA for RFLP analysis, although 21 % of the DNA had been degraded. The other alternative for preserving the DNA samples are storing in the solution containing 100 mM Tris HCl, 40 mM EDTA pH 8.0 and 0.2% SDS 11), in absolute alcohol 1,11) ,

in waterfree acetone 1) or in saturated saline containing 0.2 % sodium azide or 20 % DMSO 11). In Indonesian conditions, the most possible preservation method is to store at 0 to - 20 °C. This study showed that the preservation up to 6 weeks at - 20 °C kept the DNA from the degradation process. On the other hand, the examination delays up to 6 weeks are safe enough for the DNA samples as long as the samples are stored at - 20 °C,

The DNA extraction from postmortem tissue is basically the same as other DNA extraction methods 16). Although the DNA precipitate from the tissue sample is usually more than those from the blood and seminal sample, the quality of DNA is usually lower than the others 16). In this condition the establishment of the best extraction method is important. This study investigated Bar and Kirby methods because these methods are quite different in chemical consumption and simplicity. Kirby method 11) is basically the conventional method, consisting of cell digestion by proteinase K, phenol extraction, dialysis, butanol concentration and ethanol precipitation. The application of proteinase K in this method is performed on high concentration because it is based on the amount of samples. On the other hand, the Bar method 1) is more simple than Kirby method because it only consists of cell digestion by proteinase K, extraction by phenol and methylene chloride and ethanol precipitation. The reaction volume of Bar method is 1 ml, thus this method only needs 2 ml tube and consumes a few chemicals. The comparison of these methods showed that Kirby method resulted in more DNA yields at the rate of 1.39 times. Macroscopically, the DNA recovered by Kirby method was white, while those by Bar method often showed the yellowish to brownish discoloration. We did not explore this impurity, however probably it was the same brown impurity found in the mummy's DNA, which was suspected to be Maillard products reducing sugars 19). The presence of the brownish pellet after drying the DNA suggested that the DNA recovery will be low because of bound protein 11) and this study had confirmed this matter. To remove the impurity, a special technique by sucrose gradient was suggested 19). The re-extraction of the DNA from the phenol chloroform step could also reduce the impurity. The other possibility of this impurity

is pyridine hemochromogen derived from heme b (in hemoglobin, myoglobin, cytochrome b, cytochrome P450 and catalase) which has the absorbance peak at 556 nm. This impurity does not affect the ratio OD260/OD280 and can be decolorized by Centricon treatment 12). The removal of the impurity , by adding BSA (bovine Serum albumin) or formamide, or by using Chelex 100 or Centricon 100, is essential because the heme contaminant can inhibit the PCR amplification 12).

The spectrophotometric assessment of the DNA purity showed that the DNA extracted by Bar and Kirby methods had the same purity. However, the analysis of HMW-DNA on agarose gel electrophoresis showed that Bar method provided more HMW-DNA than Kirby method (84.25 % versus 61.9 %). The DNA typing on the D1S80 locus using PCR method showed that all of these samples could be analyzed. This findings showed that Kirby method in some aspects was better than Bar method. However, since this method consumes many chemicals, the further modification may be necessary.

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