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A method for extracting DNA from hard tissues for use in forensic identification

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Abstract. With deceased and decayed bodies, personal identification is performed using hard tissue DNA, commonly extracted from bone. The quantity and quality of DNA used in the polymerase chain reaction (PCR) amplification step is critical for a successful outcome. Since enamel is the strongest tissue in the human body, it was hypothesized that teeth may preserve DNA better than bones. In the present study, porcine teeth and bone samples were exposed to a variety of environments that imitated personal identification conditions, and DNA extracted from the teeth and bone samples was compared, using a PCR amplification method. The porcine teeth and bones were exposed to 11 different conditions for 5 different time periods to imitate a series of common crime scenes. DNA was extracted by a standard phenol-chloroform method. To test DNA quality, PCR was performed with primers designed to amplify porcine β-actin (*ACTB*) and mitochondrial DNA (mtDNA) sequences. The results demonstrated that the quality of DNA extracted from teeth was greater than that extracted from bone in the following environments: Buried in sand, soaked in caustic soda and burnt with rubber. By contrast, the quality of DNA extracted from bone was greater than that extracted from teeth when samples were buried in soil or submerged in water. There was no discernable difference in the quality of DNA extracted from bones and teeth in several environments, including being submerged in seawater, soaked in sulfuric acid, left in open air, and stored at 4, -20 and -80˚C. Additionally, the results suggested that PCR using mtDNA primers performed better than that using ACTB primers. Finally, it was indicated that components of seawater may inhibit PCR amplification. The preliminary data reported here may provide basic guidelines for selecting the optimum source of DNA in each case.

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Abbreviations: ACTB, β-actin; HV, hypervariable; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; STR, short tandem repeat

Key words: bone, teeth, DNA, polymerase chain reaction, environments

Introduction

When performing personal identification by short tandem repeat (STR) analysis, the source of the DNA extracted from the cadaver varies depending on the time passed since the point of expiration (1-5). If fewer than 24 h have passed, DNA is obtained from white blood cells. For bodies found within 2-5 days of expiration, cartilage is typically used for extracting DNA; if more than 5 days have passed, bone and other hard tissues are the final option (1,3).

The use of poor quality DNA is a common challenge for STR analysis (2,3,6). Tissue necrosis, for example, results in DNA degradation and shortening (7). Necrosis generally occurs more readily in soft tissues than in hard tissues (1,3,8). For STR analysis, the most commonly used hard tissue is bone, which preserves DNA more effectively than soft tissue (1,9). However, extracting DNA from bone can be a difficult and time-consuming process (1,3,9).

There are instances when bodies cannot be personally identified using DNA extracted from bone. The environment at the crime scene and the time elapsed since passing are the largest contributing factors to identification failure (2,6,9). This failure in personal identification can contribute toward the grief of relatives by disrupting aspects, including inheritance management and life insurance claims. These failures may also affect lawsuits in the case of murder.

Since enamel is the hardest tissue in the human body (10), we hypothesized that teeth may preserve DNA better than bone. In the present study, experiments used porcine teeth and bone as mimics for human teeth and bone. The environments of known crime scenes were imitated using 11 different conditions and various timeframes, the longest being 1 year.

Polymerase chain reaction (PCR) was performed as the first step of STR analysis and DNA quality was evaluated using primers for porcine β-actin (*ACTB*) and mitochondrial DNA (mtDNA). Additionally, inhibitors of PCR were tested in certain select conditions.

Materials and methods

Experimental design. In the current study, the two hard tissues of bones and teeth were compared. Porcine teeth and bones were selected since they are easily obtained and have macroscopic features similar to human teeth and bones. The environmental conditions for teeth and bone exposure were designed by imitating the conditions of various crime scenes based on biological evidence. DNA quality was measured by the presence or absence of PCR products. PCR was performed using primers for porcine *ACTB* to mimic human STR analysis and porcine mtDNA to mimic human mitochondrial analysis. The porcine *ACTB* PCR product size was designed to be longer than the longest PCR product size derived from human STR analysis (2). Similarly, the mitochondrial PCR product size was designed by considering the length of the mitochondria variable region (11). Additional details of the experimental design are depicted in Fig. 1.

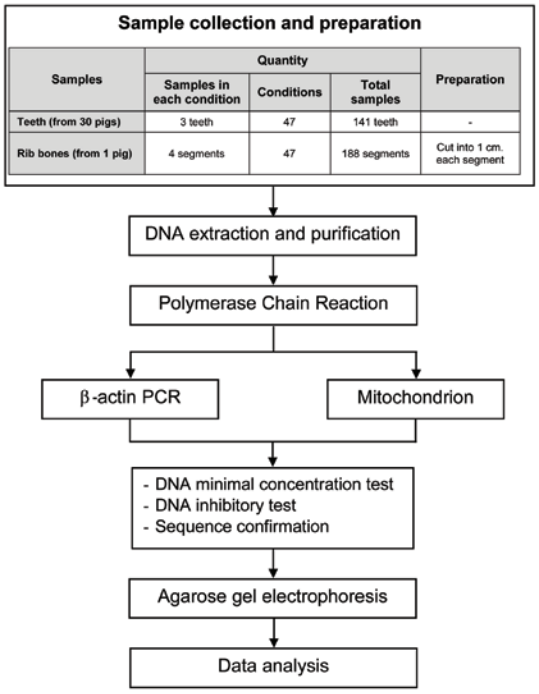

Figure 1. Flow chart of experiments. ACTB, β-actin; mtDNA, mitochondrial DNA.

Sample selection and preparation. Porcine teeth and bones were obtained from remains obtained from a local farm in Phanat Nikom, Chonburi; a period of approximately 8-10 h transpired from slaughter to the extraction of samples from the carcasses. A total of 141 teeth were extracted from 30 animals; three teeth were selected randomly for testing in each environmental condition. For the bone samples, four rib bones were extracted from one animal and cut into 188 pieces of 1 cm thickness; four bone samples were randomly selected for testing in each environmental condition. As a positive control for the PCR reactions, a soft tissue sample of porcine muscle was segmented and stored at -20˚C until DNA extraction. All animals were 3 to 4 months of age.

Environmental conditions and timelines for the samples. The samples were exposed to 11 different environmental conditions under the normal tropical climate conditions of Bangkok, Thailand. They were left in open-air, buried in soil, buried in sand (*in situ* in Phayathai, Bangkok, Thailand), submerged in water (*in situ* in Bang Sue Canal, Phayathai, Bangkok, Thailand), submerged in seawater (*in situ* at Samaesan Pier, Sattahib, Chonburi, Thailand), soaked in caustic soda (Merck KGaA, Darmstadt, Germany), soaked in sulfuric acid (Merck KGaA), burnt with rubber (30 pieces of rubber sized 0.2 x 5.0 x 5.0 cm each, duration 3 h), and stored at 4, -20 and -80˚C, respectively (Table I). For the majority of the conditions, DNA was extracted after 1 week, 1, 3 and 6 months, and 1 year. For the samples soaked in caustic soda or sulfuric acid, DNA was extracted after 1 day, 1 week and 2 weeks. For the samples burnt with rubber, DNA was extracted immediately following the experiment.

DNA extraction. Following removal of each sample from its respective environmental condition at the designated time, it was crushed with a hammer and stored overnight at 50˚C with a lysis buffer (0.75 mol/l NaCl, 0.024 mol/l EDTA, pH 8.0) containing 10% sodium dodecyl sulfate and 20 mg/ml proteinase K (Sigma-Aldrich; Merck KGaA). DNA was extracted by a standard phenol-chloroform extraction protocol (12,13). Following alcohol precipitation, the DNA samples were air-dried at room temperature and resuspended in 50 μl distilled water. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer (ND-1000 Spectrophotometer; NanoDrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Next, the isolated genomic DNA was eluted in distilled water. The optical density 260/280 ratio was greater than 1.8, which is acceptable for DNA purity and PCR (14). The detailed procedure is presented in Fig. 2.

Primer design and PCR conditions. Primers to amplify ACTB were designed from the Sus scrofa ACTB gene, partial coding sequence (NCBI sequence ID: DQ452569.1; https://www.ncbi.nlm.nih.gov), which imitates the product of the STR marker PCR reference from FBI CODIS Core STR Loci, retrieved from https://strbase.nist.gov/coreSTRs.htm (April 1, 2017). The length of the ACTB PCR product in the presesnt study was 475 bp, longer than the maximum PCR length of 464 bp, retrieved from https://strbase.nist.gov/fbicore.htm (April 1, 2017). The primer sequences were porcine *ACTB* forward, 5'-AGATCGTGCGGGACATCAAG-3' and porcine *ACTB* reverse, 5'-GAGAGAAGCCCGACTGAGC-3'.

The mitochondrial primers were designed from the Sus scrofa mtDNA sequence from mitochondrial isolate Y1, complete genome (sequence ID: KT372134.1), which imitates the human mitochondrial marker used to confirm the maternal lineage when personal identification has failed (15). The length of the PCR product was 357 bp, which covers the hypervariable (HV) regions (HV1:16024-16365 and HV2:73-340). The primer sequences were porcine mtDNA forward,

Table I. Results of *ACTB* and mtDNA PCR from bone and teeth DNA under various conditions.

Condition	*ACTB* PCR		mtDNA PCR	
	Bone	Teeth	Bone	Teeth
Positive control	+	+	+	+
Left in open-air				
1 w	+	+	+	+
1 m	+	+	+	+
3 m	+	+	+	+
6 m	+	+	+	+
1 y	-	-	+	+
Buried in soil				
1 w	+	-	+	-
1 m	+	-	+	-
3 m	-	-	+	-
6 m	-	-	-	-
1 y	-	+	+	+
Buried in sand				
1 w	+	+	+	+
1 m	+	+	+	+
3 m	+	+	+	+
6 m	-	-	-	+
1 y	-	+	-	+
Submerged in water				
1 w	+	+	+	+
1 m	-	-	+	-
3 m	-	-	-	-
6 m	-	-	-	-
1 y	-	-	-	-
Submerged in seawater				
1 w	+	+	+	+
1 m	-	-	-	-
3 m	-	-	-	-
6 m	-	-	-	-
1 y	-	-	-	-
Soaked in caustic soda				
1 d	-	+	-	+
1 w	Samples could not be collected			
2 w	Samples could not be collected			
Soaked in sulfuric acid				
1 d	+	+	+	+
1 w	-	-	-	-
2 w	Samples could not be collected			
Burnt with rubber	-	+	-	+
Stored at 4˚C				
1 w	+	+	+	+
1 m	+	+	+	+
3 m	+	+	+	+
6 m	+	+	+	+
1 y	+	+	+	+
Stored at -20˚C				
1 w	+	+	+	+
1 m	+	+	+	+
3 m	+	+	+	+
6 m	+	+	+	+
1 y	+	+	+	+
Stored at -80˚C				
1 w	+	+	+	+
1 m	+	+	+	+
3 m	+	+	+	+
6 m	+	+	+	+
1 y	+	+	+	+

+, amplified; -, unamplified; w, week; m, month; y, year; ACTB, β-actin; mtDNA, mitochondrial DNA.

5'-GGAGCAGTGTTCGCCATTAT-3' and porcine mtDNA reverse, 5'-TTCTCGTTTTGATGCGAATG-3'.

The *ACTB* and mtDNA PCR reactions contained 1X PCR buffer, 200 mM dNTPs, 0.2 mM of each primer, 0.5 U Taq DNA polymerase (Qiagen, Inc., Valencia, CA, USA), and 50 ng template DNA. The polymerase was activated by incubation at 95˚C for 15 min, followed by 40 cycles at 95˚C for 1 min, 68˚C for 1 min and 72˚C for 1 min, and a final extension at 72˚C for 7 min. DNA extracted from porcine muscle and distilled water were used as the positive and negative PCR controls, respectively. Following amplification, the PCR products were separated by gel electrophoresis using a 2% agarose gel in Tris-borate-EDTA buffer, and then stained with SYBR-Green nucleic acid gel stain (GelStar™; Lonza Group, Ltd., Basel, Switzerland) for 40 min at room temperature.

Designation of positive and negative DNA quality depended upon visualization of the PCR products. The confirmation of a PCR product from a minimum of one sample of a specific environmental condition resulted in that sample type being counted as positive for the condition. The results from a selection of the DNA samples were confirmed by direct sequencing of the PCR products (16) to verify the sequence accuracy.

Tests to determine the minimal DNA concentration and PCR inhibitory factors. The sensitivity of the ACTB and mtDNA PCR reactions was tested by 10-fold serial dilution of the DNA template from 10 to 0.001 ng/μl. Certain unamplified DNA samples were randomly tested for the presence of inhibitor components by mixing positive control DNA and individual unamplified DNA templates equally in PCR reactions.

Results

PCR amplification comparing DNA samples purified from porcine bones and teeth. Certain PCR samples were submitted for direct sequencing. The sequencing results revealed 100% similarity to the original template DNA. All results are summarized in Fig. 3 and Table I.

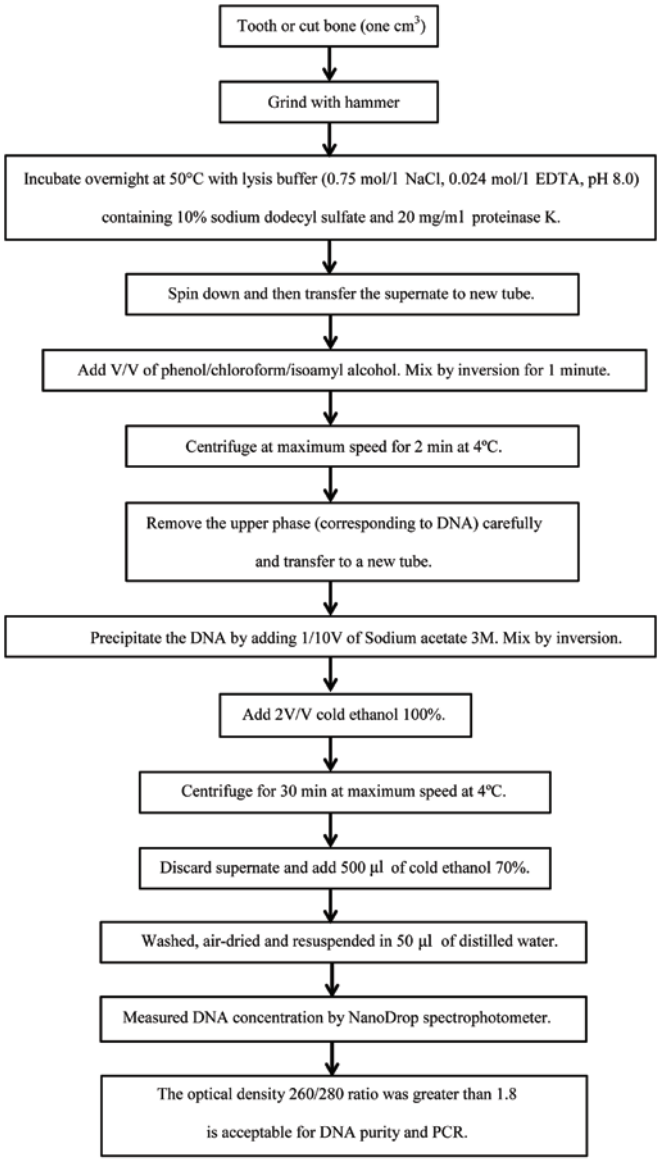

Figure 2. Standard operating procedure of DNA extraction from hard tissue for forensic identification.

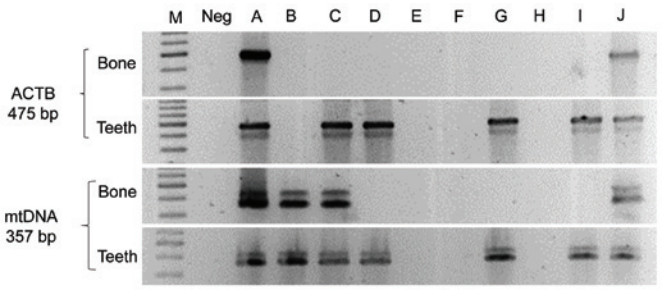

Figure 3. Comparison of DNA samples purified from porcine bones and teeth following *ACTB* and mtDNA polymerase chain reaction. M, 100 bp ladder; Neg, distilled water; A, positive control; B, left in open-air for 1 year; C, buried in soil for 1 year; D, buried in sand for 1 year; E, submerged in water for 1 year; F, submerged in seawater 1 year; G, soaked in caustic soda for 1 day; H, soaked in sulfuric acid for 1 week; I, burnt with rubber; J, stored at -80˚C. ACTB, β-actin; mtDNA, mitochondrial DNA.

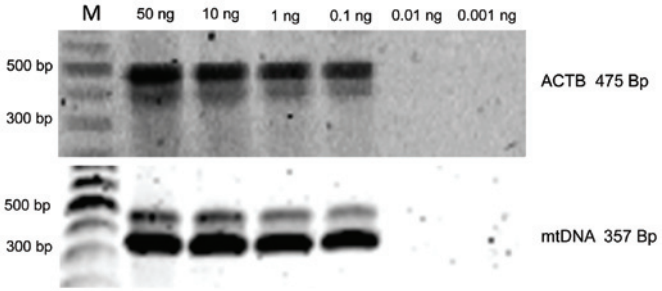

Figure 4. DNA minimal concentration test. ACTB, β-actin; mtDNA, mitochondrial DNA.

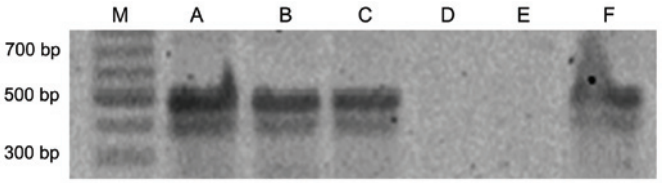

Figure 5. Inhibitory test (β-actin polymerase chain reaction). M, 100 bp ladder; A, positive control; B, tooth buried in soil for 1 week; C, tooth submerged in water for 1 month; D, tooth submerged in seawater for 1 month; E, bone submerged in seawater for 1 month; F, bone soaked in sulfuric acid for 1 week.

For the samples left in open-air, submerged in seawater or soaked in sulfuric acid, DNA extracted from bones and teeth had similar quality, and was capable of PCR amplification for both *ACTB* and mtDNA. For the samples buried in soil for 3 months or less, bones displayed positive PCR results for ACTB (samples buried ≤1 month) and mtDNA (samples buried ≤3 months). When buried in soil for 6 months, bones and teeth were negative for *ACTB* and mtDNA PCR products. Notably, at 1 year, DNA extracted from teeth amplified *ACTB* and mtDNA PCR products, and DNA from bones amplified mtDNA PCR products (Table I).

For samples buried in sand, DNA extracted from bones and teeth amplified *ACTB* and mtDNA PCR products during the first 3 months; however, for the samples buried in sand for longer periods, only DNA extracted from teeth demonstrated PCR amplification. For samples submerged in water and seawater, DNA extracted from bones and teeth demonstrated amplification of *ACTB* and mtDNA PCR products after 1 week. Over longer periods of time, PCR amplification was only observed for mtDNA from bones submerged in water for 1 month (Table I).

For samples soaked in caustic soda and burnt with rubber, *ACTB* and mtDNA PCR products were amplified only for teeth. DNA from samples soaked in caustic soda for 1 or 2 weeks could not be collected due to curd formation. Meanwhile, samples soaked in sulfuric acid were destroyed after 1 week (Table I).

Samples stored at cold temperatures (4, -20 and -80˚C) demonstrated the best DNA preservation, with positive results for *ACTB* and mtDNA PCR throughout the experimental observation period (Table I).

Comparison of ACTB and mtDNA PCR. PCR amplification of mtDNA yielded a greater rate of positive results compared with that of *ACTB* in multiple situations, including from bone

and teeth samples left in open-air for 1 year, from bones buried in soil for 3 months, from bones buried in soil for 1 year and from bones submerged in water for 1 month (Table I).

Testing minimal DNA concentrations and PCR inhibitors. The minimum amount of DNA necessary for PCR amplification of *ACTB* and mtDNA templates was 0.1 ng/μl (Fig. 4). Additionally, certain DNA samples that failed to amplify PCR products were selected to test for the presence of reaction inhibitors. To perform this test, DNA from samples that failed to amplify in the initial PCR reaction was mixed with positive control DNA for an additional PCR amplification. The samples that subsequently demonstrated PCR amplification were: Teeth buried in soil for 1 week, teeth submerged in water for 1 month, and bones soaked in sulfuric acid for 1 week. By contrast, the DNA extracted from teeth and bones submerged in seawater for 1 month still exhibited no amplification, indicating the presence of a PCR inhibitor with the DNA template (Fig. 5).

Discussion

The specific circumstances of crime scene environments in which bodies are discovered can contribute toward difficulties in personal identification due to the deterioration of DNA quality (17,18). In the present study, crime scene scenarios were simulated by artificially creating environments that imitated known crime scene conditions. DNA extracted from porcine teeth and bones was substituted, and PCR amplification of porcine *ACTB* and mtDNA sequences was performed to imitate human personal identification by PCR.

The efficiency of these PCR reactions was tested, and the minimal concentration of DNA required for PCR amplification was determined as 0.1 ng/μl. In the samples where amplification from either bones or teeth was not possible, it is likely that the DNA had deteriorated. This DNA degradation, in particular, samples buried in soil or submerged in water were observed in our study. Another potential explanation is the presence of reaction inhibitors. The results of the present study demonstrated that DNA extracted from samples submerged in seawater contained components that interfered with PCR amplification. Seawater commonly contains ions, including calcium, magnesium, sodium, potassium, chloride, sulfate and nitrate, as well as other inorganic trace elements including lead, copper, arsenic and manganese (19). The inhibitory effect of divalent ions (Ca^{2+} and Mg^{2+}), in particular, are considerable due to sensitivity to *Taq* polymerase activity (19). Further study of this phenomenon should focus on inhibitor reduction through the improvement of DNA preparation techniques.

Mitochondrial DNA has a higher copy number than nuclear *ACTB* DNA (11), and is therefore easier to amplify. The present results confirmed this characteristic for DNA extracted from samples left in several conditions, including open-air, in which mtDNA was amplified but not *ACTB* after up to 1 year.

There were certain unexpected results. PCR products were unable to be amplified using DNA extracted from samples buried in sand or soil for 6 months, but reactions were successful using DNA from samples buried for 1 year. This observation has been previously reported in the literature, and reasoned as being due to dried tissue resulting in improved DNA quality and thus easier PCR amplification (20).

For several of the simulated crime scene environments, DNA extracted from teeth resulted in improved PCR amplification, compared with DNA extracted from bones. One reason may be that tooth enamel contains more inorganic material than bone (21,22), resulting in increased protection of intracellular DNA. We hypothesized that teeth could be used as an alternative DNA source in certain situations. For example, in the case of DNA extracted from samples buried in sand for 1 year, teeth displayed results superior to bone. The results also confirmed that DNA extracted from all samples stored at cold temperatures (4, -20 and -80˚C) was well preserved after 1 year. More stable storage temperatures may aid in maintaining DNA stability at low temperatures (23).

The present study has certain limitations. Firstly, a basic phenol-chloroform DNA extraction technique was employed. There are now more advanced technologies for DNA extraction available, including spin-column DNA purification and magnetic bead DNA isolation (24,25). Use of these methods may improve the quality and quantity of DNA extracted (17,25). However, these kits are often cost prohibitive in certain locations; therefore, the results of the present study may aid with the decision to amplify bone or teeth DNA in developing countries. Secondly, levels of DNA degradation due to the exposure conditions used in agarose gel electrophoresis were not checked. Only the quantity of DNA by nanodrop spectrophotometry and the quality of DNA by PCR amplification were assessed. Degradation of the DNA may have impacted on the success of PCR amplification and should be considered in future applications.

In conclusion, the present study imitated various environments in tropical areas where unidentified bodies are commonly found, with similarities in microbial composition, temperature, humidity and pH. Therefore, the results of the present study may prove useful for countries in tropical areas as a preliminary reference for sample selection in various situations. Additionally, the use of primers for porcine *ACTB* DNA imitated the use of STR markers in personal identification, though in cases where *ACTB* DNA amplification was not possible, primers to amplify mtDNA appeared beneficial in the identification of a maternal lineage relationship, due to increased mtDNA copy number. Personal identification using DNA is a process important to law enforcement in matters including inheritance management and life insurance claims, as well as being used as evidence for lawsuits in the case of murder.

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Red Cross Society and Chulalongkorn University (Bangkok, Thailand).

Availability of data and materials

All data generated and/or analyzed during this study are included in this published article. The authors permit reuse of all illustrations in this study.

Authors' contributions

The experiments were conducted and designed by JS, AM and NK. Sample collection and laboratory experiments were performed by JS, TSo, TA, TSr, PP, AS and NK. The results were analyzed and interpreted by JS and NK. JS and NK wrote the manuscript. NK and AM reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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