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Brief Communication

Evaluation of forensic examination of extremely aged seminal stains

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ABSTRACT

The results of forensic tests, such as semen identification and short tandem repeat (STR) analysis of extremely aged seminal stains from unsolved sex crimes can provide important evidence. In this study we evaluated whether current forensic methods could be applied to seminal stains that were stored at room temperature for 33–56 years ($n = 2$, 33 years old; $n = 1$, 41 years old; $n = 1$, 44 years old; $n = 1$, 56 years old). The prostatic acid phosphatase (SM-test reagent), microscopic (Baecchi stain method) and semenogelin (RSID™ Semen Laboratory Kit) tests were performed as discriminative tests for semen. In addition, the mRNA levels of the semen-specific proteins semenogelin 1 (*SEMG1*) and protamine 2 (*PRM2*) were investigated. STRs were analyzed using the AmpFlSTR® Identifiler™ PCR Amplification Kit. All samples were positive in the prostatic acid phosphatase and semenogelin tests, and sperm heads were identified in all samples. The staining degree of the aged sperm heads was similar to that of fresh sperm. Although *SEMG1* mRNA was not detected in any sample, *PRM2* mRNA was detected in three samples. In the STR analysis, all loci were detected in the 33-years-old sample and five loci were detected in the 56-years-old sample. We confirmed that current forensic examinations – including STR analysis – could be applied to extremely aged seminal stains. These results could be useful for forensic practice.

1. Introduction

The seminal stains left at a crime scene can become important evidence in sexual crimes because the discrimination of semen is useful for understanding the details of a crime and DNA typing can identify a suspect. Presently, real-time PCR assays based on the mRNA levels of semen-specific genes [1–5] and immuno-chromatography assays of semen-specific proteins [6] are considered novel semen identification methods in addition to traditional methods, such as the acid phosphatase test and microscopic test. Moreover, DNA typing methods, such as analysis of short tandem repeats (STR), have replaced ABO blood typing in personal identification. Thus, recently, the semen examination methods used have changed significantly. The results of these newly developed techniques might differ from those of traditional methods.

In sexual crime cases in a country that has a long statute of limitation system or that does not have a statute of limitation system, reinvestigation of seminal stains might be performed using current techniques several decades after the crime occurred. Kido et al. reported that the nine STR loci were typed from seminal stains stored for up to 25 years using a commercial kit [7]. However, this kit is no longer manufactured. Therefore, in extremely aged (several decades) seminal stains, current semen investigation methods based on discrimination and DNA typing, and their usefulness for forensic practice, have not been reported. If proven to be applicable to extremely aged seminal stains, the present examination methods could be useful in criminal trials.

We used 33–56-years-old seminal stains stored at room temperature for seminal discrimination examinations and DNA typing and evaluated the results in terms of forensic usefulness.

2. Materials and methods

2.1. Samples

We used five seminal stains adhered to cotton gauze and stored at room temperature ($n = 2$, 33 years old; $n = 1$, 41 years old; $n = 1$, 44 years old; $n = 1$, 56 years old). The seminal stain used as the positive control sample comprised fresh semen adhering to cotton gauze collected from one donor, who gave informed consent. This study was approved by the Ethics Committee of Saitama Medical University.

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2.2. Discrimination test for semen

The prostatic acid phosphatase test, microscopic test and semenogelin test were performed as discrimination tests for semen.

The prostatic acid phosphatase test as the preliminary semen discrimination test was performed using SM-test reagent (Wako, Osaka, Japan) according to the manufacturer's protocol. The prepared SM-test reagent was dropped directly onto a piece of gauze and then incubated at room temperature. We defined a positive result as purple coloration observed by the naked eye 5 min after incubation.

The sperm head was confirmed by microscopy using the Baecchi stain method. The sperms on a 0.5 × 0.5-cm piece of gauze were exuded by 20 μL of distilled water. The exudation solution was immobilized on glass slides and then stained with Baecchi stain reagent.

The semen-specific protein semenogelin was placed on a 0.5 × 0.5-cm piece of gauze and detected using RSID™ Semen Laboratory Kit (Independent Forensics, Lombard, IL, USA) according to the manufacturer's protocol.

2.3. DNA/RNA extraction and cDNA synthesis

For the DNA and the total RNA extractions, we used a 1 × 1-cm piece of gauze. The DNA was extracted and purified using QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and eluted in 60 μL of water. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and DNase I digestion was performed on the RNA extracts using RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using a High-capacity RNA-to-cDNA™ Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, by addition of 9 μL of RNA to a total reaction volume of 20 μL.

2.4. Real-time PCR

To investigate expression of semen-specific factors, the semenogelin 1 (*SEMG1*; Hs00268141_m1, 82 bp) and protein protamine 2 (*PRM2*; Hs00172518_m1, 89 bp) genes were selected; 18S rRNA (*18S*; Hs99999901_s1, 187 bp), actin-beta (*ACTB*; Hs99999903_m1, 171 bp) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1, 124 bp) were chosen as endogenous controls. All primers and probes were used with a commercially designed kit (Life Technologies). Real-time PCR was performed in 20-μL reaction mixtures containing 2× TaqMan® Fast Advanced Master Mix (Life Technologies), 20× TaqMan® Gene Expression Assay (primers and probe; Life Technologies) and 2 μL of cDNA. PCR amplification was performed using the StepOne-Plus Real-Time PCR System (Life Technologies) programmed for 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Ct detection threshold was determined automatically with the software incorporated in the StepOne-Plus Real-Time PCR System. When the Ct difference (ΔCt) between a target gene and the endogenous control was determined to be within the quantification range determined by preliminary experiments, mRNA expression was defined as positive.

To quantify DNA fragments of several lengths, DNA extracts were measured using a KAPA Human Genomic DNA Quantification and QC Kit (Kapa Biosystems, Inc., Woburn, MA, USA), which can quantify 41-, 129- and 305-bp DNA fragments, according to the manufacturer's protocol, using the StepOne-Plus Real-Time PCR System.

2.5. STR analysis

STR analysis was performed using the AmpFlSTR® Identifiler™ PCR Amplification Kit (Life Technologies) according to the manufacturer's protocol. STR alleles were detected on an ABI 310 Genetic Analyzer (Life Technologies), following by analysis using the GeneMapper™ ID software (Life Technologies). The positive threshold of peak height was defined as >150 relative fluorescent units (RFUs).

3. Results and discussion

3.1. Discrimination test for semen

The results of the prostatic acid phosphatase test, microscopic test, semenogelin test and determination of mRNA levels are shown in Table 1. All samples exhibited a positive reaction in the prostatic acid phosphatase and semenogelin tests; the sperm head was found in all samples. Additionally, the degree of staining of the aged sperm heads was similar to that of fresh sperm, indicating that these tests are applicable to extremely aged seminal stains as semen discrimination tests.

ACTB mRNA and *GAPDH* mRNA were not detected in aged samples (data not shown); however, *18S* mRNA was detected in all aged samples, and the endogenous control genes were detected in fresh seminal stains. Therefore, in this study we used the *18S* mRNA gene as the endogenous detection gene in aged seminal stains. *SEMG1* mRNA was not detected in aged samples; however, *PRM2* mRNA was detected in three samples. *SEMG1* is a seminal-vesicle- and prostate-specific gene [8] and *PRM2* is a spermatozoa-specific gene [9]. Due to the disulfide cross-links in the protamine surrounding the sperm, sperm DNA is not released unless treated with a reducing agent [10]. The difference between the *SEMG1* and *PRM2* mRNA results might be due to cell membrane stability – the spermatozoon cell membrane is firmer than that of the prostate gland. Lindenbergh et al. reported detection of *SEMG1* and *PRM2* mRNA in 28-years-old seminal stain samples [11]. However, in our results, *PRM2* mRNA was detected in the 56-years-old sample. Sakurada et al. considered dryness and light as important factors in mRNA degradation [3]. Because the samples

Table 1
The results of the prostatic acid phosphatase test, microscopic test, semenogelin test and mRNA levels.

Sample name (storage duration)	SM test	Baecchi stain	RSID-semen	mRNA (mean ± SD)*		
				SEMG1	*PRM2*	*18S*
56 years	+	+	+	ND	36.20 ± 0.57	24.68 ± 0.16
44 years	+	+	+	ND	ND	29.23 ± 0.04
41 years	+	+	+	ND	ND	29.63 ± 0.17
33 years-1	+	+	+	ND	32.86 ± 0.43	31.55 ± 0.12
33 years-2	+	+	+	ND	33.08 ± 0.50	31.32 ± 0.30
Fresh	+	+	+	26.94 ± 0.03	30.35 ± 0.25	14.41 ± 0.03

ND: Not detected.
* The mean and standard deviation (SD) in Ct value were calculated from 3 replicates.