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TECHNICAL NOTE

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Applicability of Nanotrap Sg as a semen detection kit before male-specific DNA profiling in sexual assaults

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Abstract A commercially available semen detection kit, Nanotrap Sg, which employs a one-step detection test based on immunochromatographic assay for the semenogelin protein, was evaluated for profiling male-specific DNA in sexual assault casework samples. While semen diluted with phosphate-buffered saline held and kept at 4°C for 1 week showed a relatively strong signal intensity with Nanotrap Sg, the signal intensity was decreased by dilution after storage at 4°C or freezing and thawing repeated more than three times. The reproducibility of Nanotrap Sg was tested on a total of 174 sexual assault casework samples from three forensic laboratories using intra- and interassay and no variation was observed in the semenogelin (Sg) signal. The positive signal ratio was 12.6% higher for prostate-specific antigen immunochromatographic membrane tests than Nanotrap Sg. Although spermatozoa were not confirmed in 61 (35%) out of 174 samples, Sg-positive signals could be detected from 41 (67%) of the 61 samples. Female genetic profiles could be observed in 95% of the samples, which tested negative for Sg on the Nanotrap Sg test, but no male genetic profiles could be observed. These results suggest that Nanotrap Sg can positively identify samples containing male DNA even in the absence of detectable intact spermatozoa. Further, Sg-positive signals identified samples for which male-specific DNA profiling could be performed, even if no sperm could be detected from the sample. The potential of Nanotrap Sg for identifying forensic samples with male-specific DNA was clearly demonstrated.

Keywords Forensic sciences · Semen · Semenogelin · Immunochromatography · Sexual assault

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Introduction

DNA analysis of evidence collected from victims of sexual assault, often involves analysis of semen that was contaminated by various body fluids of female, damaged and degraded, and may have dried into a stain. Detection of semen from stains is a very important confirmatory test before making an analysis of gamete DNA. Therefore, developing a more objective method for the detection of semen is of utmost urgency.

Recently, methods for isolating male specific messenger RNA for capturing male-derived cells were reported [1, 2]. Although these methods would be expected to identify male DNA in profiling mixed (male and female) body fluids, special equipment with high running costs and high time requirements is needed to carry out the analyses.

Two semenogelin (Sg) genes coding for Sg-I (52 kDa) and Sg-II (76 kDa) proteins are located in the q12–q13.1 region of human chromosome 20 [15, 23]. These two genes are highly homologous and comprise three exons of 4,164 and 8,224 bp, respectively. These proteins are well known to play a role in spontaneous semen coagulation after ejaculation and Sg-I inhibits sperm motility [9, 17]. Recently, Sato et al.[18, 19] reported Sg to be a more suitable marker for semen identification than prostate-specific antigen (PSA) and subsequently, a sensitive membrane-based Sg test was developed and made commercially available for forensic use in Japan in 2004. We tested for the presence of Sg in forensic samples using this new immunochromatography method.

Materials and methods

Specimens

Forensic casework samples (174 samples) including soiled and stained clothing, victims' body surfaces, tissue paper samples, carpet and bed sheet samples, vaginal swabs, anal swabs, and liquid saliva samples provided from the Scientific Crime Laboratory (Kanagawa Prefectural Police, Yokohama, *Japan*), the Carabinieri Scientific Investigation Department of Rome (Rome, *Italy*), and the Forensic Science Laboratory (Hiroshima Prefectural Police, HQ, Hiroshima, *Japan*) were used in this study. Each specimen was stored at −80°C until required for use.

Native Sg-II and recombinant Sg-II were given from the department of Urology, St. Marianna University School of Medicine, Kawasaki, Japan.

Confirmatory test for semen identification

Sg and PSA were detected by Nanotrap Sg (Rohto Pharm, Osaka, *Japan*) and Seratec PSA Semiquant (Seratec Diagnostica, Gottingen, Germany) or the PSA-Check 1 (VEDALAB, Alencon, France) kits, respectively. The immunochromatographic membrane test for PSA was assayed according to the manufacturer's instructions. Spermatozoa microscopically were examined by simple staining with fuchsin acid and methylene blue.

Sg membrane test assay

The construction and properties of Nanotrap Sg were described previously in detail by Sato et al. [20]. The one-step immunochromatographic system employs monoclonal and polyclonal antibodies against recombinant Sg-II (76 kDa), which had been synthesized in insect cells using baculovirus [14]. For stain or liquid specimens, the samples were extracted or diluted with extraction buffer from the kit; for example, a 5×5-mm^2 piece of fabric was extracted with 500 μl of extraction buffer. A Sg concentration sufficient to yield a distinct red-purple colored vertical line contrasting against a white background in the immunoreactive probe zone within 20 min in the sample was used. The color intensity of Sg signal peaked for the 2,000-fold dilution of semen and then disappeared completely at dilutions in excess of 400,000-fold (Fig. 1). Sg-positive signals on Nanotrap Sg were observed in previous work on Sg immunotesting [20].

For serial dilutions of blood with extraction buffer, blood could not be developed with the Nanotrap Sg, which could not be used for dilutions up to eightfold because the fluid samples are still highly viscous (Fig. 2a).

For blood and semen diluted with extraction buffer at 16- and 1,000-fold, respectively, and also for blood added to semen at a ratio of 50:1, a positive Sg signal was produced with the same color intensity as a 10,000-fold dilution of semen (Fig. 2b).

Fig. 1 Intensity of immunoreactive signal on Nanotrap Sg. Semen samples and saline were applied to the test stick of Nanotrap Sg. *Upper*: semen, 400,000-fold dilution; *middle*: semen, 2,000-fold dilution; and *lower*: saline

DNA profiling

DNA samples were isolated using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with slight modifications according to [25]. In addition, samples were isolated using Chelex-100 by the method of Walsh et al. [24]. A total of 15 or 10 loci, in addition to Amelogenin, were analyzed using the AmpFLSTR Identifiler PCR Amplification or the AmpFLSTR Profiler Plus PCR Amplification (Applied Biosystems, Foster City, CA, USA) STR kits, respectively. For samples testing positive for female DNA by these two methods, Y chromosome-specific STRs were analyzed using the Power Plex Y system (Promega, Corporation, Madison, WI, USA). DNA typing was carried out on the ABI Prism 3100 or 310 Genetic Analyzer (Applied Biosystems) and the results obtained from GeneScan electropherogram were analyzed by GeneMapper v.3.2 or the 3.1 Software (Applied Biosystems).

Results

Property of antigenic activity in Sg and PSA

Nanotrap Sg is an immunochromatography membrane test constructed using monoclonal and polyclonal antibodies against recombinant Sg-II (76 kDa) [20]. To compare the immunoreactive signal of purified Sg-II obtained from seminal plasma and recombinant Sg-II assayed by Nanotrap Sg, the respective signals were quantified by the method of Sato et al. [20]. Native Sg-II showed a threefold higher signal than recombinant Sg-II for the immunoreactive band of Nanotrap Sg (Fig. 3). On the other hand, antigenic activity of Sg and PSA in seminal plasma detected by immunochromatography membrane tests showed PSA antigenic activity to be eightfold higher than that of Sg (Table 1).

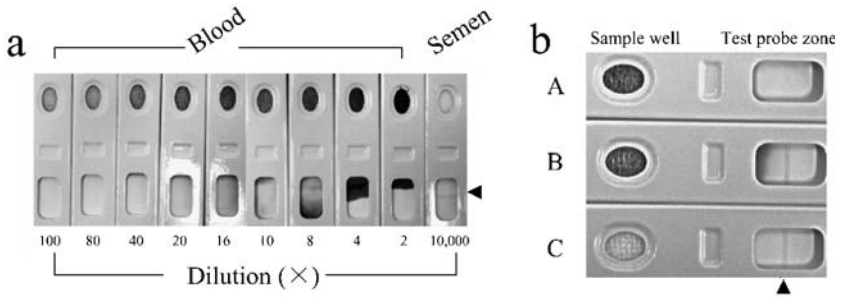

Fig. 2 Chromatogram patterns on Nanotrap Sg. **a** Blood was diluted with the extraction buffer from 2 to 100-fold and then applied to the test stick of Nanotrap Sg. **b** *A* blood (16-fold dilution), *B* mixture (blood 16-fold dilution and semen 1,000-fold dilution) applied at a ratio of 50:1, and *C* semen (10,000-fold dilution). *Arrows* show Sg positive signal

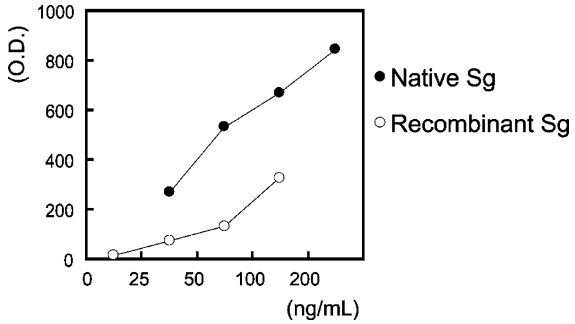

Fig. 3 Immunoreactivity of native Sg and recombinant Sg on Nanotrap Sg

Stability of antigenic Sg and PSA activities after storage

Storage procedures for semen and or extracted Sg antigens from stains were also evaluated. Ejaculated semen was immediately frozen until use and after thawing, the semen was stored under various conditions and the signal intensities of Sg and PSA were compared. Although PSA signal intensity showed no decrease under any of the tested conditions, the intensity of the Sg signal decreased for semen stored at 4°C for 1 week and diluted 50,000-fold with phosphate-buffered saline and for samples that were subjected to freezing and thawing repeated more than three times (Table 2).

Assay variation of Nanotrap Sg on confirmatory test for semen in casework samples

The results of 174 forensic casework samples subjected to semen detection using Nanotrap Sg are shown in Table 3. To examine reproducibility, two areas from a single stain were tested independently by one or two individuals (intra- and interassay, respectively). The positive or negative results from the intra- and interassay coefficients of variation were 1.2 and 3.7%, respectively, reflecting the composition of each of the specimens. The positive signal ratio of samples was 12.6% higher for PSA immunochromatographic membrane tests than for Nanotrap Sg in the 174 casework samples in this study (Table 3). Although positive and negative ratios for the Sg test showed similar results to that for sperm detection, Sg-positive signals could be detected from 41 (67%) of the negative sperm samples (n=61). Male-specific DNA profiles were detected in 113 (73%) of the Sg-positive samples (n=154) and no male DNA was detected from the Sg-negative samples.

Discussion

We first describe the applicability of Nanotrap Sg for detection of semen in the forensic practice then its availability for practical application. Although the detection limit of Sg by Nanotrap Sg corresponds to a 200,000-fold dilution of seminal plasma, Nanotrap Sg showed sensitivity from two- to fourfold times greater against native Sg than recombinant Sg [20] (Fig. 3 and Table 1). However, Sg in ejaculated semen was gradually fragmented mainly by chymotrypsin-like protease PSA [14, 17]. Many fragments of Sg present in semen during this degradation may be trapped with the polyclonal and monoclonal antibodies against recombinant Sg-II (76 kDa) in the Nanotrap Sg assay. Although Sg could be successfully detected in semen stains stored for 5 years at room temperature [20], the signal from Nanotrap Sg was decreased by repeated freezing and thawing of diluted samples. This phenomenon may be due to the artificial damage incurred rather than the physiological degradation (Table 2). Although some commercially available immunochromatography tests for serum PSA may have higher sensitivity against semen than Nanotrap Sg, as demonstrated in this study, the detection limit for PSA in serum in the

Table 1 Detection limit of the antigenic activity of Sg or PSA in seminal plasma

Immunochromatography kit	Seminal plasma (dilutions)							
	10^3	10^4	10^5	2×10^5	4×10^5	8×10^5	16×10^5	32×10^5
Nanotrap Sg	+	+	$+_w$	±	(−)			
PSA-Check 1	+	+	+	+	$+_w$	±	±	±

+, $+_w$ (weak), ±, and (−) in the test of Nanotrap Sg or PSA-Check 1 indicate the visible color immunoreactive signal on the test stick described in Sato et al. [20] (Nanotrap Sg) and Sato et al. [19] (PSA-Check 1)

Table 2 Effect of various conditions to the signal intensity on the immunochromatography membrane test

Storage	Nanotrap Sg	PSA-Check 1
Dilution[a]	+	+
Dilution after semen was stored at 4°C	$+_w$	+
Semen stored at 4°C after dilution	+	+
Dilution after freezing and thawing at one time	+	+
Dilution after freezing and thawing two times	$+_w$	+

[a]Semen was diluted at 50,000 and 20,000-fold and then applied to Nanotrap Sg and PSA-Check 1, respectively

manufacturer's instruction is 4 ng/ml [19] (Table 1). In addition, positive signals on PSA immunochromatography tests were often false positive results due to the high sensitivities of these tests. PSA is well known as a widely used clinical marker for tumors of prostate cancer [10] and it was also recently found to be in breast tumors [6, 27]. Furthermore, PSA was found in tissues and body fluids associated with pregnancy such as endometrium [5], placenta [11], maternal serum [28], breast milk [29], and amniotic fluid [13, Sato et al., unpublished data]. In addition, PSA activity is present in urine of males from early infancy up to more than 10 years of age [19, Sato et al., unpublished data] and in the urine of females taking oral contraceptives or after sexual intercourse [4, 12]. Therefore, androgen-dependent PSA can be considered neither male-specific nor semen-specific and consequently, these biochemical properties of PSA make it unsuitable for identifying semen in forensic casework [7]. In addition, commercially available reagents for forensic use should be applicable to multiple types of specimens, rather than only single type (i.e., serum) [8]. However, Sg was also recently found in the serum of lung cancer patients suffering for adenocarcinoma or squamous cell carcinoma of lung [3]. Therefore, to correctly evaluate the results of positive signals for Nanotrap Sg, the clinical history of suspects and victims including smoking history should be known.

The formation of semen from seminal vesicle fluid and prostatic fluid and the number of spermatozoa is never uniform, thus, the resulting stains or forensic evidence are also not expected to be uniform. However, the present study showed that Nanotrap Sg can cover the wide range of seminal stains based on the results of reproducibility tests (Table 3) due to the higher contribution of the seminal vesicle fluid than prostatic fluid (60–70% vs 15–20% in semen) [20] resulting in a mean Sg concentration that is more than twice that of PSA (19:5.5 mg/ml) [20, 26].

Although STR typing in sexual assault cases targets the male gamete DNA and the high discrimination power can directly identify the suspect, the longer spermatozoa remains in the reproductive tract, the microscopic examination of spermatozoa becomes more difficult because most sperm lose their tails after ejaculation into the reproductive tract and become difficult to identify by morphological studies [18]. In addition, microscopic examination is adversely affected by the age of the stain and varies with the density of the deposited ejaculate. Also, it is well known that while the concentration of spermatozoa in healthy men is almost always greater than 100 millions per ejaculation, it is never uniform in a year and at times, sperm are absent from the semen [16]. In the present microscopic study, Sg-positive signals could be detected from 67% of the samples in which spermatozoa could not be observed (Table 3). In addition, the male-specific DNA could be identified in 73% of the Sg-positive forensic

Table 3 Confirmatory test for semen in 174 casework samples

Test		Sg/PSA	Signal	Profile	*n* (%)
Intra-assay		Sg	Yes		30 (1.2)
Interassay		Sg	No		30 (3.7)
Concordance study		Sg	Yes		119/174 (68.4)
			No		55/174 (31.6)
		PSA	Yes		141/174 (81)
			No		33/174 (19)
Microscopic study					
Sperm	Yes 113/174 (65)	Sg	Yes		113/113 (100)
	No 61/174 (35)	Sg	Yes		41/61 (67)
DNA typing study		Sg	Yes	M	113/154 (73)
				F	0/154 (0)
				M & F	32/154 (21)
				ND	9/154 (6)
			No	M	0/20 (0)
				F	19/20 (95)
				M & F	0/20 (0)
				ND	1/20 (5)

M Male, *F* female, and *ND* not detectable

samples and in 21% of the samples containing mixed female and male DNA; a positive Sg signal suggests the detection of spermatozoa and consequently Y chromosomal STRs, which indicates that male-specific DNA profiling should be possible in the mixed DNA [21]. These results suggest that male DNA profiles are obtainable not only from gametes but also from seminal epithelial cells and leukocytes containing B and T cells [1, 16, 22]. Although 73% of male-specific DNA profiles were detected from Sg-positive samples, no male DNA was detected from Sg-negative samples.

In conclusion, Nanotrap Sg is a useful and convenient tool for identifying samples for male-specific DNA testing in forensic cases involving sexual assaults. Furthermore, Nanotrap Sg has the advantage of being convenient and can potentially be used not only for forensic laboratory analysis but also in clinical diagnosis for victims of rape/child abuse and domestic violence. In addition, fieldwork can be easily carried out at the crime scene due to the ease of use and because no special equipment is required for the use of the Nanotrap Sg test kit.

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