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Transferrin (TF) Typing from Semen Stains Using Isoelectric Focusing and Immunoblotting: Correlation of TF Types Among Blood, Semen, Urine, and Vaginal Secretion

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ABSTRACT: We describe a method for obtaining nondistorted and reproducible transferrin (TF) typing from liquid semen and semen stains. Isoelectric focusing of TF isoproteins on polyacrylamide gel (IEF-PAGE, pH 4 to 6.5) was accomplished using a 0.5 mm thick gel. The separated isoproteins were then visualized by immunoblotting with TF-specific antibody. Pretreatment of semen samples with neuraminidase enhanced the TF band resolution. The method was reliable, sensitive and simple, with a high resolution. When maintained at room temperature, laboratory-prepared semen stains were TF-typable for up to at least 50 weeks. The TF types in semen stains were correlated with the types found in the corresponding blood and urine samples. TF typing could therefore provide an additional discriminant characteristic in the forensic examination of semen stains. An evaluation of TF typing by IEF-PAGE and immunoblotting was also performed on casework samples submitted to our laboratory.

KEYWORDS: criminalistics, transferrin, body fluids, isoelectric focusing, immunoblotting, semen identification

Transferrins (TF) are iron-binding glycoproteins found in the physiological fluids and cells of mammals [*1*]. Serum TF produced by the liver is the major iron-binding and -transport glycoprotein present in serum and physiological fluids [*2*]. A testicular form of TF is also synthesized and secreted by cultured rat Sertoli cells [*3,4*]. These are testicular epithelial cells present in the seminiferous tubules, which interact with developing germinal cells. The formation of tight junctional complexes between Sertoli cells results in the formation of a functional blood/testis barrier [*5*]. The secreted testicular TF has been

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found to have iron-binding ability, immunological activity and a molecular mass similar to those of serum TF [*1*].

Genetic polymorphism of TF has been detected in serum by isoelectric focusing, and six common phenotypes of TF are known to be controlled by three co-dominant alleles, *TF*C1, TF*C2* and *TF*C3*, in Caucasians [*6,7*], whereas three common phenotypes are controlled by two alleles, *TF*C1* and *TF*C2* in Japanese [*8,9*]. Recent data suggesting selection at the TF locus have led to speculation about the involvement of TF in reproduction. Alleles at the TF locus are associated with differences in fertility or spontaneous abortion: a high incidence of the *C2* gene in mothers with a previous history of spontaneous abortion [*10*]; a significant increase in the frequency of *C2* among extremely premature infants [*11*]; an increased frequency of *C3*, instead of *C2*, in couples with first-trimester recurrent spontaneous abortion [*12*].

Although the most common markers used for individualization of semen in semen stains have been ABO and phosphoglucomutase-1 (PGM1) systems, the interpretation of the results is sometimes problematic [*13*]. The authors' experience using the ABO and PGM1 systems for body fluid-stain typing in a casework situation has shown that the value of the evidence obtained, in most cases, is very low in comparison to the effort expended in carrying out the investigation. Consequently, there is a need for a more effective system of discrimination. Serum TF isoproteins are very useful for discriminating different TF phenotypes in bloodstains [*14*]. If the TF isoprotein type expressed in serum could be used as a genetic marker in semen and semen stains, this would be of considerable value for investigation of crime materials.

Yamaba et al. [*14*] have described a preliminary survey assessing the correlation between serum and seminal TF, in which semen and blood samples from 103 men were examined. In their series of experiments, no instances of "non-correlation" were encountered. Consequently, we have pursued further correlation studies to evaluate the use of seminal TF in a casework situation. In this line of work, semen stains are encountered not only in the form of whole uncontaminated semen but also as mixtures with other body fluids. The most common contaminant of semen in this context is vaginal secretion, either on vaginal swabs or in stains as a consequence of vaginal drainage after sexual intercourse. Although semen is sometimes found mixed with saliva, urine and other contaminants during the course of crime investigation, it is not practical to investigate semen stains other than the most commonly encountered form. Consequently, this paper describes experiments carried out on stains of pure semen and semen/vaginal secretion mixtures.

Materials and Methods

Preparation of Samples for Analysis

Blood Samples—Venous blood was taken into tubes containing heparin.

The blood samples were stored at +4°C before being centrifuged and the plasma separated. These plasma samples were stored at −80°C until determination of TF grouping.

Collection of Semen—Donors supplied samples obtained by masturbation or by withdrawal during sexual intercourse. The ejaculate was collected in clean, wide-mouthed plastic containers with screw caps. These samples were presented at the laboratory within 12 h of collection and immediately frozen at −80°C prior to subsequent handling.

Collection of Vaginal Swabs—During examinations for gynecological insufficiency, five vaginal swabs were taken from the vaginal fornix. These swabs were stored immediately

at −80°C until required for testing. The samples were taken on standard wooden-spilled cotton wool swabs.

Preparation of Semen Stains—The experimental design of the survey required either three or four separate stains of whole semen to be made from each sample. Each semen sample (1 mL) was mixed thoroughly after thawing and then pipetted onto clean cotton sheeting. The stains so made were approximately 30 cm^2 in area. These were hung up to air-dry, divided into five separate groups and stored in separate paper envelopes at −20°C, +4°C, in a humid chamber at +4°C, at room temperature and at +37°C until required for TF grouping.

Preparation of Vaginal Swab Extracts—The cotton wool tip of one swab from each female donor was removed and extracted in two drops of distilled water (ca. 60 μL). This was carried out in the upper compartment of the extraction device described previously [*16*]. After centrifugation, the swab extract was divided into two portions. One of these was tested for acid phosphatase activity by dropping onto clean filter paper, followed by one drop of the phosphatase reagent used for screening [*13*]. The remainder of the swab extract was tested for TF grouping [*8,9*].

Extraction of TF Protein from Semen Stains—A piece of semen-stained cotton sheeting measuring 5 by 5 mm (contained about 0.83 μL liquid semen) and 15 μL of each solution for TF extraction were employed for each test according to the micro-extraction method described in our previous paper [*16*]. As the diluent of the extracts, a 1% (w/v) solution of bovine serum albumin was used in order to prevent TF denaturation.

Preparation of Urine Samples—Urine samples were concentrated, dialyzed and finally freeze-dried, as described previously [*17,18*]. A 1% solution of the freeze-dried material, corresponding to about 100-fold concentrated urine, was used for TF typing.

Method of TF Typing

Polyacrylamide gel isoelectric focusing (IEF-PAGE) was performed in thin-layer gel, pH 4-6.5, as described previously [*8*]. Gels measuring 0.5 (thickness) by 90 (width) by 120 (length) mm were prepared using the following materials: 1.4 mL acrylamide-bisacrylamide (19.4%, w/v; 0.6%, w/v), 1 mL distilled water, 2.3 mL sucrose-glycerol (20%, w/v; 10%, v/v), 280 μL Ampholine 4-6.5 (Pharmacia LKB, Uppsala, Sweden), 5 μL *N,N,N′,N′*-tetramethylethylenediamine, and 40 μL 1.2% ammonium persulfate. Wicks were formed from strips of filter paper and soaked in the electrode solution: 0.04 *M* glutamic acid in 0.5 *M* H_3PO_4 at the anode and 0.1 *M* β-alanine at the cathode. Each sample was applied to the gel with a plastic applicator at a distance of 10 mm from the cathode wick. A Multiphor apparatus (Pharmacia LKB) was employed to run the gel at V_{max} 1000 V, I_{max} 10 mA, and P_{max} 5 W for 4 h under cooling at +12°C. Transfer of proteins onto a Durapore membrane strip (Millipore, Bedford, USA) was carried out by capillary blotting as described in our previous paper [*8*].

Visualization was achieved using anti-TF (rabbit, Dakopatts, Glostrup, Denmark) as a primary antibody, peroxidase-conjugated anti-rabbit immunoglobulin (goat, Bio-Rad, Richmond, USA) as a secondary antibody, and H_2O_2 with 3,3′-diaminobenzidine as a substrate [*9*]. The extract, urine solution or diluted semen (1:4 with distilled water) was treated with an equal volume of 5 units/mL *Clostridium perfringens* neuraminidase (Sigma, St. Louis, USA) before electrophoresis [*8,9*]. Five microliters of the digest was used for IEF-PAGE analysis. Blood and urine samples were analyzed by the previous method [*8*].

Method of Seminal TF Quantitation

Quantitation of seminal TF was performed by rocket immunoelectrophoresis followed by amplification of the immunoprecipitates using peroxidase-labeled antibody essentially according to the method of Kjærvig Broe and Ingild [*13*]. For this, 0.7 mL antibody-free agarose (0.6 mL 0.9% agarose dissolved in Tris-barbital buffer, pH 8.6, I = 0.02, containing 0.2% calcium lactate and 0.1% sodium azide and 3% polyethylene glycol 6000) was poured onto the lower 1 by 5 cm of a 5 by 6-cm hydrophilic plastic film (Marysol, Tokyo, Japan). After congelation, 3.15 mL 0.9% agarose, 0.35 mL 30% polyethylene glycol 6000 and 15 μL of 1:30-diluted rabbit anti-TF were mixed, and the mixture was poured onto the upper 5 by 5 cm of the plastic film. Nine application wells, 2 mm in diameter, were cut out in the antibody-free gel. After dilution (1:5), 2-μL semen samples were deposited in the wells. After completion of electrophoresis (8.9 V/cm, 3.5 h, 5°C), the following procedure was carried out: 1) Press the gel for 15 min, 2) wash in 0.9% saline for 20 min, 3) press for 15 min, 4) incubate for 10 min in 0.9% saline containing 0.05% Tween 20 (ST), 5) incubate for 60 min in 1:400-diluted peroxidase-labeled goat anti-rabbit immunoglobulin in ST, 6) press for 15 min, 7) wash in 0.9% saline for 20 min, 8) press for 15 min, 9) immediately before use, dissolve 2 mg of 3-amino-9-ethylcarbazole (AEC) in 0.5 mL dimethyl sulfoxide and mix with 5 mL 0.05 *M* CH_3COOH/CH_3COONa, pH 5.0, then add 3 μL H_2O_2. Incubate the gel in the mixture for 15 min, 10) wash in distilled water for 10 min, and 11) dry at room temperature.

Quantitation of TF was performed by measurement of rocket heights from the upper edge of the antibody-free gel to the apex of the rocket. A plot of the height *versus* the initial known concentration (μg/mL) of TF (standard serum X908, Dakopatts) was then made. The amounts of TF in the semen samples were read from the standard curve.

Results

Quantitation of Seminal TF

The levels of seminal TF were estimated from 110 samples by quantitative rocket immunoelectrophoresis with amplification of the immunoprecipitates by peroxidase-labeled antibody. This assay is shown in Fig. 1 to illustrate the technique. The mean value of the seminal TF was determined to be 27.22 ± 19.98 μg/mL (range, 2.7 to 111.3 μg/mL). This mean value did not agree with other studies [*15,16*] (70 to 100 μg/mL). The standard deviation for the levels of TF was as high as 70% of the mean value. Comparison of the intra- and interindividual variances showed that the major component of variation was intraindividual. This means that there is considerable variation in the quantitative expression of TF from ejaculate to ejaculate.

Liquid Semen Survey

The TF type of 110 Japanese male donors was determined from both blood and semen samples, and the results agreed with the donor's TF types in each case, as described for our preliminary survey [*14*]. These samples were groupable into 5 types: C1, 69; C2-1, 30; C2, 7; B Yonago-C1, 2; D Chi-C1, 2. The IEF-PAGE patterns of seminal TF in each type were identical to those of serum TF. This supposition was supported by the finding that a 1:10 to 20 (vol:vol) mixture of serum and semen of type C1 gave a single isoprotein band indistinguishable from that of type C1 serum on the gel. Another mixture of serum and semen of type C2 also gave a single band.

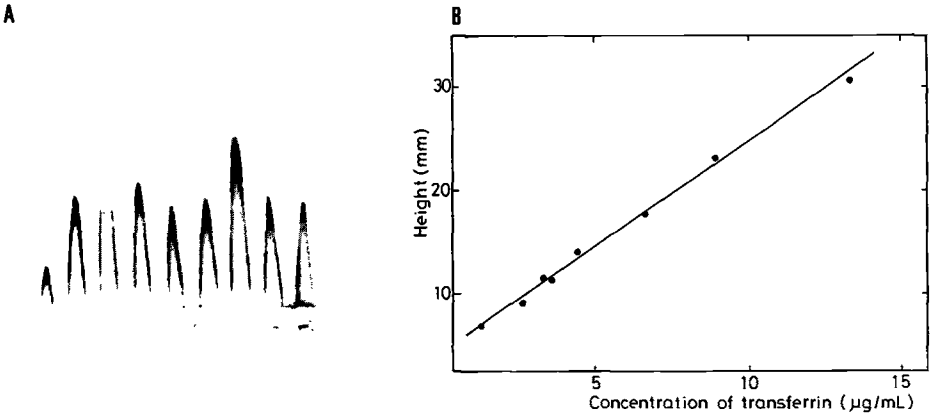

FIG. 1-A,-B—*Rocket immunoelectrophoresis assay for transferrin. A-Photographic representation of the assay pattern. Antigen: 2 μL samples of transferrin reference (X908, Dakopatts) diluted in electrophoresis buffer containing 1% bovine serum albumin (application well 1, 2.7 ng; well 2, 13.4 ng; well 3, 26.7 ng transferrin, from left to right), and 5-fold-diluted semen samples from six different men (wells 4 to 9). Antibody: Rabbit anti-human transferrin (A061, Dakopatts), 0.02 μL/cm² gel. B-The standard curve for quantitation of the seminal transferrin. Antigen: 2 μL samples of transferrin reference (X908, Dakopatts) diluted serially in electrophoresis buffer containing 1% bovine serum albumin, the concentration of which is given in the figure. Antibody: the same antiserum used in FIG. 1-A. The rocket height from the upper edge of the lower part of the gel with application wells to the apex of the rocket was plotted against the known amount of transferrin. The amounts of seminal transferrin were estimated from the standard curve.*

Effects of Solutions on TF Extraction

Reducing reagents (2-mercaptoethanol and dithiothreitol) at different concentrations and physiological saline had no favorable effect on extraction of TF protein from the aged stains, which were kept for 5 weeks at room temperature. Next, the effects of several detergents for extraction of TF protein were examined (Table 1). CHAPS at 0.25% gave the most effective extraction from the aged stains, and therefore it was employed for each subsequent test throughout this study. Urea at 0.3-1.0 *M* or Tween 80 at 0.5% (v/v) was also considerably effective for TF extraction.

Semen Stain Survey

Details of the results are shown in Table 2. The possibility of typing TF isoprotein in semen stains aged up to 50 weeks was examined under four different conditions. Every

TABLE 1—*Effects of various solutions at different concentrations for extraction of transferrin from semen stains stored at room temperature for 5 weeks.*[a]

Solution	2%	1%	0.5%	0.25%	0.1%	0.05%
CHAPS[b]	+	+ +	+ + +	+ + +	+	−
Tween 20	+	+ +	+	+	−	−
Tween 80	+	+ +	+ +	+	−	−
Nonidet P-40	+	+ +	+ +	+	−	−
Triton X-100	+	+ +	+ +	+	−	−

[a] + + +, most effective; + +, more effective; +, effective; −, effect equal to distilled water.
[b] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate ($C_{32}H_{58}N_2O_7S$).

TABLE 2—*Transferrin typing of semen stains stored under various conditions.*[a]

Weeks	−20°C	+4°C	Room Temp.	+37°C	Humid, +4°C
1 (day)	+	+	+	+	+
1	+	+	+	+	+
4	+	+	+	+	+
9	+	+	+	+	+
15	+	+	+	+	+
27	+	+	+	+	+
50	+	+	+	−	+

[a] +, correct identification of TF types; −, inconclusive or no result.

Artificial semen stains tested contained five different subtypes, C1, C2-1, C2, B Yonago-C1, and D Chi-C1. These stains of three common types, C1, C2-1 and C2, were made from semen samples from more than five different males, respectively.

sample stored at −20°C, +4°C and room temperature was phenotypable without false interpretation after dry or humid storage conditions for the entire duration of the experiment. However, most of samples kept at 37°C for 50 weeks were difficult to type because of broad and indistinct band formation with a higher background. Some examples of the results obtained from aged semen stains stored under these conditions are shown in Fig. 2.

Detection Limits for Semen Stains

We also determined the detection limits for semen stains: these limits were determined on the basis of the area of piece of stains used, volume of solution for TF extraction, volume of sialidase solution, sample volume for IEF-PAGE, and the maximum dilution of the sample solution required for TF typing (see Materials and Methods). Under four dry storage conditions, except at −20°C, a slow decrease in the antigenic activity of TF was observed during the course of storage. The detection limit had dropped to 1/20 of the level for fresh stains after 50 weeks of dry storage at room temperature (Table 3), and similar results were obtained after dry storage at +4°C and +37°C. The amount of semen stain required in order to type heterozygotes (C2-1, D Chi-C1 and B Yonago-C1) was approximately twice that for homozygotes (C1 and C2). It proved possible to determine TF types from 50-week-old stains made from about 0.8 μL of semen and kept at room temperature.

Correlation of TF Types Among Semen, Blood and Urine

TF types were examined in 37 sets of semen, serum and urine samples supplied from the same 37 male donors. The TF type of a male donor was determined from the donor's semen and urine samples, and the results agreed with the donor's types in each case (Table 4).

Vaginal Swab Survey

Five female donors, ranging in age between 21 and 40 years, and who had not had intercourse immediately before the study, provided semen-free swabs without menstrual contamination. In all instances, the antigenic activity was deemed sufficiently intense for determination of TF type, and the vaginal TF type corresponded to the TF type of each subject's blood and urine (C1, 4; C2-1, 1). However, the TF content of each swab per unit weight of protein was considerably lower than that of semen.

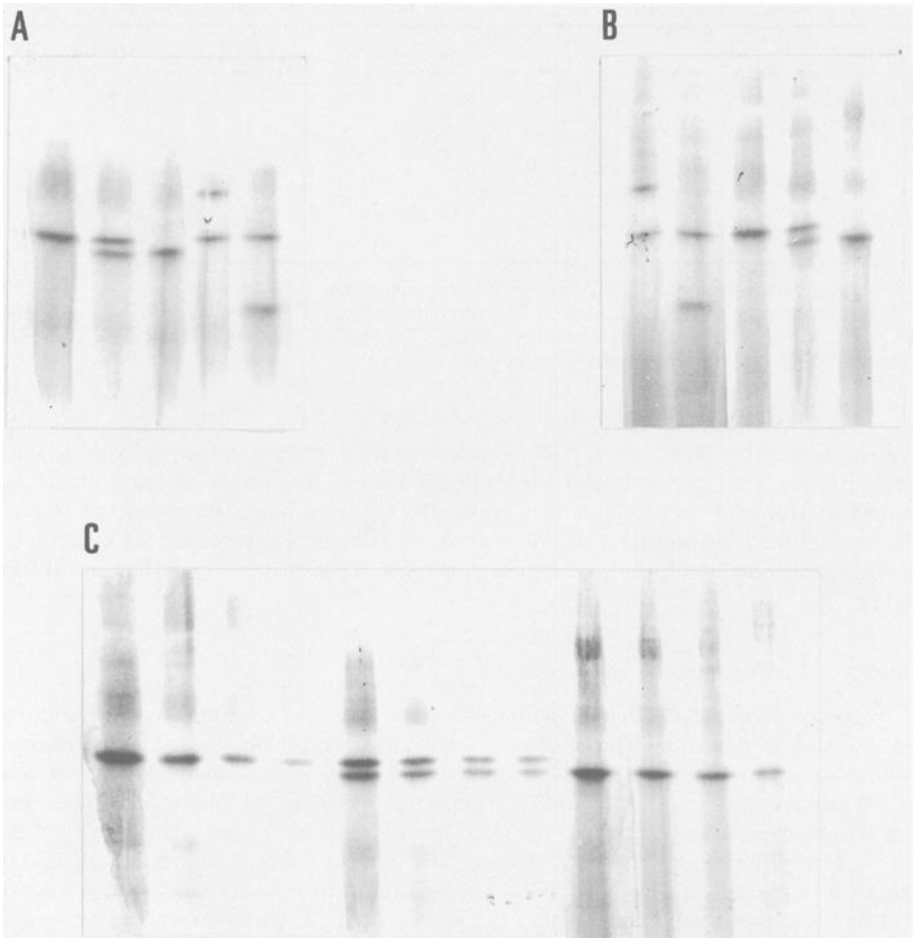

FIG. 2-A,-B,-C—*Isoelectric focusing patterns of transferrin subtypes from semen stains stored at 37°C for 4 weeks* (A: *lane 1, C1; lane 2, C2-1; lane 3, C2; lane 4, B Yonago-C1; lane 5, D Chi-C1 from left to right), semen stains stored at room temperature for 27 weeks* (B: *lane 1, B Yonago-C1; lane 2, D Chi-C1; lane 3, C1; lane 4, C2-1; lane 5, C2), and semen stains stored at room temperature for 15 weeks* (C: *lanes 1, 2, 3 and 4, C1 stains equivalent to 800, 400, 200 and 100 nL of liquid semen, respectively; lanes 5, 6, 7 and 8, C2-1 stains equivalent to 1600, 800, 400 and 200 nL of liquid semen; lanes 9, 10, 11 and 12, C2 stains equivalent to 800, 400, 200 and 100 nL of liquid semen). Anode is at the top of each panel.*

Survey on Items from Casework

Details of a very limited number of items from routine casework in our laboratory are given in Table 5. Unfortunately, the time elapsed between the offense and the swab being taken and other detailed informations on the victims, suspects, and crime scenes were unknown in each case. A swab (case 1) from a woman of type C1 gave a band pattern that was interpreted as C1 mixed with faint C2 or C2-1 semen. The suspect in this case could not be typed because no blood or urine sample was available for examination.

TABLE 3—*Minimum detection limits obtained from semen stains of different transferrin types stored at room temperature.*[a]

Weeks	TF phenotype		
	C1	C2-1	C2
1 (day)	20	40	20
1	30	50	30
2	30	50	30
4	100	200	100
9	200	400	200
15	200	400	200
27	200	400	200
50	400	800	400

[a]nL of liquid semen required for TF typing from the stains. The values were averaged from the results of five test samples in all cases. Other experimental conditions are described under Materials and Methods.

TABLE 4—*Correlations of transferrin types among semen, blood and urine.*[a]

TF types	No. of pairs of semen, blood and urine samples tested		
	Semen	Blood	Urine
C1	20	20	20
C2-1	10	10	10
C2	5	5	5
B Yonago-C1	1	1	1
D Chi-C1	1	1	1

[a]37 volunteer men each supplied a set of semen, blood and urine samples.

TABLE 5—*Transferrin types in items from casework.*

	Items	AP[a]	Sperm[b]	Blood-stained	TF-type	TF-type[c] Victim	TF-type[c] Suspect
Case 1	Swab of vaginal material	+	+	−	C2-1	C1	NT[d]
Case 2	Swab of vaginal material	+	+	−	C1	C1	C2
Case 3	Swab of vaginal material	+	−	+	C2-1	C2-1	C1
Case 4	Semen stains	+	+	−	C1	C2	NT[d]

[a]Acid phosphatase activity: +, positive; −, negative.
[b]Detectable microscopically: +, present; −, not present.
[c]Determined by serum or urine samples.
[d]Not tested.

Discussion

The mean value of seminal TF determined from 110 samples was 27.22 ± 19.98 μg/mL. This value was about 1/3 to 1/4 of that estimated by other workers [*15,16*]. The difference may have been due to the differences in the TF determination methods; rocket immunoelectrophoresis in this study and single radial immunodiffusion in others.

Transferrin (TF) subtypes are very valuable in terms of discrimination potential [*8*], but the TF levels in semen are reported to be close to the limits of detection by conventional TF typing [*15*], and some concentration step may be required prior to analysis [*16*]. The combined technique of IEF-PAGE and immunoblotting using a TF-specific antibody produced a 50 to 100 fold increase in sensitivity as compared with conventional detection using protein staining, and we were able to type TF from all the seminal samples (range of TF content: 2.7 to 111.3 μg/mL), because we succeeded in overcoming a major quantitative barrier preventing analysis.

Our previous work [*9,14*] showed that there was a good correlation between the TF types of semen and corresponding blood and urine samples. The present work extends these previous findings. Consequently, semen and blood samples from a total of 110 individuals (C1, 69; C2-1, 30; C2, 7; B Yonago-C1, 2; D Chi-C1, 2) have now been examined. No examples of non-correlation in TF typing between corresponding samples have been found. Moreover, all individuals showed sufficiently detectable TF bands in the semen. The surveys described in the present paper were designed to simulate some of the problems encountered by the practising forensic biologist in a casework situation, where one is required to individualize body fluids and their mixtures using genetic markers. In this respect, the surveys were designed to test the validity of the TF typing technique in these situations.

The influence of storage conditions on the time limit for detection of TF types was examined (Table 2). Every sample stored at −20°C, +4°C, and room temperature was typable without false interpretation after at least 50 weeks of dry storage. However, temperature during storage was the major constraint affecting detectability, whereas other factors such as air humidity had very little influence under the present experimental conditions. In another experiment, semen (1 μL) of type C2 was added to vaginal swabs from a type-C1 donor, and incubated in a humid chamber at +37°C to approximate in vivo conditions. Pieces were removed from the swab every 6 h and typed. The volume of semen was chosen in order to produce band patterns of optimum antigenicity, and it was found that all of this antigenicity had appeared sufficiently even after 24 h (data not shown). From this work we feel that the length of time that has elapsed between intercourse and swabbing should not be an important criterion in deciding whether TF typing should be attempted. This is striking contrast with PGM1 typing [*13*].

According to our experience, most of the proteins such as TF and C6 in aged stains were difficult to extract with water or saline only and redissolved [*14,17*]. Therefore we examined the solution used for this purpose (Table 1). CHAPS at 0.25% was the most effective among several detergents and other solutions tested, improving and increasing the efficiency of detectability of TF types by several-fold as compared with distilled water.

We also determined the limits of detection of semen stains. Under five storage conditions, except at −20°C, a slow decrease in the antigenicity of TF was observed during the course of storage. Though the detection limit had dropped to 1/20 of the level for fresh stains after 50 weeks of dry storage at room temperature, and storage at +4°C and +37°C, it proved possible to determine TF types from 50-week-old stains made from about 0.8 μL of semen (Table 3). The amount of semen stains required in order to type heterozygotes was approximately twice that for homozygotes. In practice, stains of this volume would be applicable in routine casework.

In all of five semen-free swabs tested, the TF types were established, and matched the donor's type. These results, therefore, showed that delicate care is needed when typing semen and interpreting the band patterns on vaginal swabs using TF polymorphism, even though semen contains greater amounts of TF protein than vaginal secretion. Investigation of vaginal swabs for seminal TF can yield information only when the woman is C1 (or C2) type, and when type C2 (or C1) components are detected on a semen-positive swab, these can be attributed to the semen present. In such an event, the semen on this swab

could be from a C2 (or C1) or C2-1-type man. In all other pairings, the woman's TF type would mask the seminal TF type.

Only three instances of TF typing of actual case-swabs submitted to the laboratory were tested (Table 5). In the case 1-swab it might be considered that the victim was C1 type, and the type C2 component of the suspect was detected on the vaginal swab. This could be attributable to semen which could have originated from a C2 or C2-1 type man. In the case 2-swab it was possible to ascribe a TF type corresponding to the TF type of the victim's blood. In this case, the TF activity detected could have originated from the victim's own secretions, owing to an insufficient amount of TF from the suspect's semen, or from other C1 type offender, that is, the suspect might be excluded from the true perpetrator. TF grouping system should be used routinely in the forensic investigation of crime scene material, after it has undergone rigorous evaluation including a survey of larger casework samples. The initial hope that the use of the ABO and PGM1 blood group systems for the individualization of semen in body fluid stain mixtures could be superseded by using seminal TF as a genetic marker must wait the results of further inquiry.

It has been reported that a combination of IEF-PAGE and subsequent immunodetection is very important for crime investigations of genetic markers detected in body fluids [*17–22*]. We used the technique in the present study and identified the correct TF type from aged semen stains stored over a period of 50 weeks at room temperature. This demonstrates that successful and effective detection is closely dependent on the methodology used rather than the degradation of protein with time, as pointed out previously [*14*].

In conclusion, use of the TF system for individualization of semen in body fluid stain mixtures seems to offer new and more useful information to the practising forensic biologists in addition to the use of the seminal ABO and PGM1 systems as genetic markers because of the high concentration, good gene frequency and high stability of seminal TF.

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