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mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR

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ABSTRACT

mRNA profiling is a promising new method for the identification of body fluids from biological stains. Major advantages of mRNA profiling are the possibility of detecting several body fluids in one multiplex reaction and of simultaneously isolating DNA without loss of material. A reverse transcription endpoint polymerase chain reaction (PCR) method and a realtime PCR assay were established for the identification of blood, saliva, semen, vaginal secretions and menstrual blood, and were compared to conventional enzymatic and immunologic tests. The results for specificity, sensitivity and suitability to biological stains were satisfying and mRNA stability was demonstrated for up to 2-year-old stains. Two novel multiplex assays were created with the endpoint PCR primers: multiplex 1 amplifies two markers for each of the above mentioned body fluids and is suited for screening; multiplex 2 was designed for the detection of blood, vaginal secretions and menstrual blood. The results demonstrate that both endpoint PCR and realtime PCR are suitable for the identification of body fluids in forensic stains and represent an effective alternative to conventional enzymatic and immunologic tests.

1. Introduction

Identification of the biological source of a stain is an important issue in forensic casework, that may also help in predicting the success of a DNA analysis and in interpreting DNA results. In forensic practice biological stains are routinely preanalyzed with enzymatic or immunologic tests. However, there are some problems associated with these tests. Most of them are not specific, due, for example, to cross-reactions with other species or tissues. Furthermore, no such tests are available for the identification of vaginal secretions and menstrual blood. In particular, the differentiation between menstrual blood and blood due to trauma can be crucial in forensic casework, for example, in sexual assault cases.

Recently, the analysis of cell-specific mRNA expression has been proposed as a promising new technique for the identification of body fluids in biological stains. A number of markers have been identified for the forensically most relevant body fluids: blood, saliva, semen, vaginal secretions and menstrual blood [1–11].

However, RNA is notorious for its rapid post-mortem and in vitro decay, because of the ubiquitously present RNases. Quite unexpectedly, however, several reports have pointed out a high stability of RNA under controlled conditions; for example, RNA could be isolated from 15-year-old dried blood stains [12]. Even from stains that were exposed to a range of environmental conditions for up to 547 days, RNA of sufficient quality and quantity could be isolated [13]. Since casework material is often limited, an important advantage of body fluid identification by mRNA profiling is the possibility of simultaneously isolating RNA and DNA from the same piece of stain [14,15].

In the present study a reverse transcription endpoint PCR and a realtime PCR method were developed and compared to conventional enzymatic and immunologic tests for the identification of body fluids. Endpoint PCR allows the detection of a specific transcript, when present in sufficient quantity. In contrast, realtime PCR allows the detection of relative gene expression levels in different samples and in comparison to an endogenous control. The following genes, which have been reported to be expressed in a tissue-specific manner, were analyzed: porphobilinogen deaminase (PBGD) [16], β-spectrin (SPTB) [17] and hemoglobin beta (HBB) [18] for blood, statherin (STATH) [19] and histatin 3 (HTN3) [19,20] for saliva, protamine 1 and 2 (PRM1 and PRM2) [3,21] for sperm, human beta-defensin 1 (HBD1) [22] and mucin 4 (MUC4) [23] for vaginal secretions and matrix metalloproteinases 7 and 11 (MMP7 and MMP11) [2] for menstrual blood. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA were used as endogenous controls. The endpoint PCR primers were combined and tested in two multiplexes, allowing the simultaneous amplification of several markers in one PCR reaction. Specificity, sensitivity and suitability to biological stains were

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analyzed and mRNA stability was recorded over time. The menstrual marker expression was investigated for a whole menstrual cycle (days 1–28).

2. Materials and methods

2.1. Samples

Body fluids were collected from healthy volunteers. Three individuals donated blood samples, two individuals saliva samples, semen samples were prepared from a frozen aliquot of one individual, vaginal secretion samples were donated from three individuals and menstrual blood samples from 11 individuals. Unless otherwise indicated, 10 μl aliquots of fresh blood (without anticoagulation treatment) or saliva or frozen semen were pipetted on cotton swabs and dried at room temperature. Vaginal secretions and menstrual blood were collected from the vagina on sterile cotton swabs and dried at room temperature. The swabs were stored at room temperature, in a dark place, without special humidity control, for up to 2 years. Forensic stains included 13 mock samples, four stains from a German proficiency trial (GEDNAP 34) and 15 casework samples from 11 routine cases. The 13 mock samples comprised blood on wood, cardboard, tissue, cleaning cloth and from a lab bench; saliva from chewing gum, postage stamp, bottle, cigarette and fabric; semen on cotton and fabric. GEDNAP 34 stains included 10 μl saliva on a cigarette butt, 20 μl blood on a cosmetic pad, 10 μl blood mixture on cotton and 20 μl semen–saliva mixture on cotton.

2.2. RNA-extraction and reverse transcription

Special precautions were taken to avoid the degradation of RNA by the ubiquitously present RNases, namely a separate working place only for RNA, separate pipettes, RNase-free plastic ware, cleaning of the whole working area with RNaseZap (Ambion/Applied Biosystems, Rotkreuz, Switzerland) and the regular changing of gloves. Unless otherwise indicated, the whole swabs were used for the RNA extraction. Total RNA was extracted with the RNeasy Micro/Mini Kit or the AllPrep DNA/RNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol with the following adaptation for both Mini Kits: the piece of stain was placed in 345 μl RLT-buffer + 5 μl Carrier-RNA in a DNA LoBind tube (Vaudaux-Eppendorf, Schönenbuch, Switzerland). The RNase-free DNase Set (Qiagen) was used for on-column DNA digestion. RNA was eluted in 24–30 μl H_2O, from which 22 μl were immediately reverse transcribed into cDNA. For the reverse transcription reaction, random primers and Superscript III reverse transcriptase (Invitrogen, Basel, Switzerland) were used according to the manufacturer's protocol. Half of the RNA was used as RT minus control (without reverse transcriptase) to detect genomic DNA contamination. cDNA was obtained in a final volume of 20 μl.

2.3. Endpoint PCR

Primers for the amplification of gene-specific sequences were adopted from Juusola and Ballantyne [6], including PBGD, SPTB, HTN3, PRM1, PRM2, MUC4 and MMP7. The STATH- and HBD1-primers were redesigned for the multiplex, because of unsuitable annealing temperatures. Additional primer sets were designed for HBB, MMP11, 18S rRNA and GAPDH, using the Primer3 and the Roche Applied Science-Universal Probe Library Software (Table 1). It was ensured that all primers overlap exon–exon-junctions or span an intron. The forward primers were 5′-labelled with 6-FAM, VIC, HEX or NED (Table 1). 10× Buffer I and AmpliTaq Gold Polymerase (Applied Biosystems) were used for the singleplexes

Table 1
Endpoint PCR primer sequences and realtime PCR (*) assay IDs. References can be found in Section 2.

Body fluid	Gene	Primer sequence/assay ID	Dye	Size (bp)	Reference
Blood	SPTB	f: AGG ATG GCT TGG CCT TTA AT r: ACT GCC AGC ACC TTC ATC TT	FAM	247	[6]
	SPTB*	Hs00165820_m1	FAM	61	AB
	PBGD	f. TGG ATC CCT GAG GAG GGC AGA AG r: TCT TGT CCC CTG TGG TGG ACA TAG CAA T	VIC	177	[6]
	HBB	f: GCA CGT GGA TCC TGA GAA C r: ATG GGC CAG CAC ACA GAC	FAM	61	Roche
	HBB*	Hs00747223_g1	FAM	106	AB
Saliva	STATH	f: TTT GCC TTC ATC TTG GCT CT r: CCC ATA ACC GAA TCT TCC AA	FAM	93	Primer3
	HTN3	f: GCA AAG AGA CAT CAT GGG TA r: GCC AGT CAA ACC TCC ATA ATC	FAM	134	[6]
Semen	PRM1	f: GCC AGG TAC AGA TGC TGT CGC AG r: TTA GTG TCT TCT ACA TCT CGG TCT	NED	153	[6]
	PRM2	f: GTG AGG AGC CTG AGC GAA CGC r: TTA GTG CCT TCT GCA TGT TCT CTT C	FAM	294	[6]
	PRM2*	Hs00172518_m1	FAM	89	AB
Vaginal secretions	HBD1	f: CCC AGT TCC TGA AAT CCT GA r: CAG GTG CCT TGA ATT TTG GT	FAM	215	Primer3
	MUC4	f: GGA CCA CAT TTT ATC AGG AA r: TAG AGA AAC AGG GCA TAG GA	NED	235	[6]
Menstrual blood	MMP7	f: TCA ACC ATA GGT CCA AGA AC r: CAA AGA ATT TTT GCA TCT CC	VIC	240	[6]
	MMP7*	Hs00159163_m1	FAM	101	AB
	MMP11	f: GGT GCC CTC TGA GAT CGA C r: TCA CAG GGT CAA ACT TCC AGT	NED	92	Roche
	MMP11*	Hs00171829_m1	FAM	66	AB
Housekeeping genes	GAPDH	f: TCT TCA CCA CCA CGG AGA A r: AGG GGG CAG AGA TGA TGA C	HEX	72	Roche
	GAPDH*	4326317E	VIC	122	AB
	18SrRNA	f: CTC AAC ACG GGA AAC CTC AC r: CGC TCC ACC AAC TAA GAA CG	HEX	110	Roche