Forensic Science International: Genetics 6 (2012) 452–460

Contents lists available at SciVerse ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis

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ARTICLE INFO

Article history:
Received 18 February 2011
Received in revised form 22 September 2011
Accepted 24 September 2011

Keywords:
Messenger RNA (mRNA)
mRNA profiling
Body fluid identification
Forensic science

ABSTRACT

The identification of forensically relevant human body fluids through messenger RNA (mRNA) profiling is of interest to the forensic community. Previous studies have proposed several tissue-specific mRNA markers to achieve this goal. Seven markers for the following genes were selected for evaluation in this study: histatin 3 (*HTN3*) and statherin (*STATH*) for saliva, mucin 4 (*MUC4*) for vaginal secretions, matrix metalloproteinase 7 (*MMP7*) for menstrual blood, delta-aminolevulinate synthase 2 (*ALAS2*) for peripheral blood, and protamine 2 (*PRM2*) and transglutaminase 4 (*TGM4*) for semen. The expression of these markers was examined in each body fluid. All mRNA markers were present in their target body fluids. Peripheral blood and saliva showed little cross-reactivity with the selected markers. However, a high level of cross-reactivity was observed between the vaginal secretion marker MUC4 and saliva stains. Semen showed a high level of cross-reactivity with the selected markers. Co-expression of the predicted body fluid markers was detected in menstrual blood and vaginal secretion stains. The expression pattern of these mRNA markers varied through the menstrual cycle time points tested. Differences in gene expression levels and marker cross-reactivity were observed in the donors tested. Despite the presence of cross-reactivity and co-expression, each of the body fluids examined have distinct gene expression profiles, allowing for body fluid identification based on mRNA profiling.

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1. Introduction

Blood and other human body fluids are often recovered from a variety of crime scenes, sometimes in extremely low quantities. The reliable identification of human body fluid stains could be of critical value to investigators since it provides important clues into crime scene reconstruction, and contributes toward solving crimes [1,2]. Traditional serological techniques of human body fluid identification can be labor-intensive, non-specific, presumptive, or may require large amounts of sample [2–4]. Biological stains recovered from crime scenes could benefit from the development of novel and more sensitive methods for their identification. Messenger RNA (mRNA) profiling is a novel technique that has been investigated as a potential tool for human body fluid identification [5–19]. Body fluid identification by traditional serological techniques capitalizes on the differing biological components (such as sugars, proteins, and enzymes) present in each fluid [4,20]; similarly, mRNA profiling relies on the unique expression of mRNAs to identify body fluid stains. Advantages of mRNA profiling include: the identification of a broad spectrum of body fluids, a single molecular based method for virtually all body fluids, the simultaneous detection of several stains by a multiplex PCR reaction [10,12,13,17], less sample consumption as a result of parallel co-extraction of RNA and DNA [5,21,22], and the potential to automate the extraction techniques. Recent studies conducted by the European DNA Profiling Group verified that successful identification of blood stains was possible in laboratories with no prior experience with RNA and in some laboratories co-extraction of RNA/DNA was also successful, further demonstrating the utility of mRNA profiling for body fluid stain identification in forensic applications [23,24].

Identification of menstrual blood and vaginal secretions is particularly important to cases of sexual assault, where the defense could provide a non-culpable reason for the presence of the victim's DNA on the accused's clothing, such as from a bloody nose or saliva stain [9]. In these situations, the ability to identify the origin of the stain could significantly aid the investigation. Historically, identification of menstrual blood and vaginal secretion stains has been difficult due to their co-localization and the cyclic changes that occur in both fluids [20]. Recently, through the use of mRNA profiling, several groups have demonstrated the identification of both menstrual blood and vaginal secretions [6,10,11,13,16,25].

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1872-4973/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.fsigen.2011.09.007

A number of markers suitable for the identification of human body fluid stains commonly found at crime scenes have been described based on a candidate gene approach [6,8–10,12,13,17–19,23,25–30] or whole genome expression analyses [15,31]. Two critical factors that could affect the suitability of these mRNAs to serve as markers to identify body fluid stains are (1) whether they are present in any other fluid of forensic interest and (2) whether the expression level of these transcripts differs significantly in individuals. The specificity of the selected markers has been assessed, and in some cases conflicting results have been observed [11–15,17,29]. In addition, transcript expression differences within individual donors have been indirectly addressed in some studies [6,13].

The main objective of this study was to determine whether mRNA markers for the candidate gene were present in the corresponding body fluid and absent in all others. Differences in marker expression within individuals were also assessed. To achieve these goals, 12 previously identified markers (a minimum of two for each body fluid) were initially considered for evaluation for use in mRNA profiling: PRM 1 (protamine 1), PRM2 and TGM4 (semen), HTN3 and STATH (saliva), SPTB (beta-spectrin) and ALAS2 (peripheral blood), matrix metalloproteinases MMP7, MMP10 and MMP11 (menstrual blood), MUC4 [6,8–10,12,13,17–19,23,25–30] and KRT16 (keratin 16) [32] (vaginal secretions). Of these 12 markers, 5 were excluded because of the detection of high level cross-reactivity with other body fluids and double peaks around the expected size that could not be reduced. Primer sequences for the five excluded markers can be found in Supplemental Table 1. Other studies, focusing on blood-specific markers, have also suggested that the SPTB marker is not optimal for mRNA profiling [18,19,23]. The seven markers that gave consistent results during primer optimization (PRM2, TGM4, HTN3, STATH, ALAS2, MMP7, and MUC4) were selected for use in this study.

2. Materials and methods

2.1. Sample collection

A total of 33 human subjects donated body fluids for this study. Semen samples were purchased from the Fairfax Cryobank (Fairfax, VA) and included an egg-based extender (egg yolk citrate buffer with: egg yolk, glucose, sodium citrate, water, penicillin, streptomycin, tris and glycerol (approximately 7–8%)). The other four fluids were collected from consenting, anonymous donors according to the policies of the Institutional Review Board of the Federal Bureau of Investigation. Saliva was collected in sterile 15 ml BD Falcon Centrifuge conical tubes (BD Biosciences, Franklin Lakes, NJ) at least 30 min after the donor's last intake of food or drink. Peripheral blood was drawn via venipuncture (without additive) and immediately spotted onto sterile cotton-tipped swabs (Fisher Scientific, Pittsburgh, PA). Saliva, semen, and peripheral blood, also referred to as "measurable" body fluid stains, were spotted onto sterile cotton-tipped swabs in 50 μl aliquots. Samples of vaginal secretions and menstrual blood were collected directly onto sterile cotton-tipped swabs (Fisher Scientific). The volume of biological material collected for the vaginal secretions and menstrual blood samples could not be determined or normalized, and thus these samples are referred to as "immeasurable". Vaginal secretions were collected three and seven days post-menstruation. Menstrual blood was collected at any point during menstruation. The same donors contributed the menstrual blood and vaginal secretions samples. Body fluid stains were allowed to dry overnight at room temperature prior to total RNA extraction. Two to four biological replicates and two technical replicates were included for each donor. In total, 350 body fluid stain samples were used in this study (saliva, $n = 68$; semen, $n = 64$; blood, $n = 64$; vaginal secretions day 3, $n = 52$; vaginal secretions day 7, $n = 52$; menstrual blood, $n = 50$).

2.2. RNA extraction

RNA extraction was performed using the RNAqueous®-4PCR kit (Ambion/Life Technologies). Prior to the RNA extraction, the body fluid stains were denatured using a modification of a previously described procedure [9]. Briefly, using sterile techniques, the cotton section of the dried swabs were cut and placed into 1.5 ml RNase-free microcentrifuge tubes (Ambion/Applied Biosystems, Austin, TX) containing 500 μl of pre-warmed (56 °C) lysis buffer, provided in the RNAqueous®-4PCR kit, and incubated in a water bath shaker at 56 °C for 30 min. Each swab was transferred to a UV-crosslinked spin basket and centrifuged for 10 min at $8120 \times g$ to remove the remaining solution. After the denaturation step, the RNA extraction was performed per the manufacturer's protocol. Two elution steps were performed (50 μl for the first and 35 μl for the second elution) using warm (75 °C) elution solution included in the RNAqueous®-4PCR kit. All isolated RNA samples were subjected to DNase I treatment as described in the manufacturer's protocol.

2.3. RNA quantification

RNA concentrations were measured using a NanoDrop ND-1000 UV/vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Samples were measured using the RNA-40 setting (wavelengths monitored at 260 nm and 280 nm) following the manufacturer's protocol. The average concentration for each body fluid was as follows: vaginal secretions (75 ng/μl), menstrual blood (45 ng/μl), semen (4.5 ng/μl), blood (0.81 ng/μl – below instrument threshold), and saliva (5.1 ng/μl). Samples with measurements below 3 ng/μl have been shown to be inaccurate as previously determined [33].

2.4. cDNA synthesis

Reverse transcription was performed using 15 μl of RNA extract, which was incubated for 3 min at 75 °C and then placed on ice for an additional 3 min. Fifteen microliters of master mix (6 μl of 10 mM dNTPs, 3 μl of 10× first strand synthesis buffer, 3 μl of 50 μM random decamers, 1.5 μl of 20 units/μl SUPERase-In, and 1.5 μl of 100 units/μl M-MLV reverse transcriptase, per reaction – Applied Biosystems/Ambion) were then added to each RNA sample, and the reaction was incubated at 42 °C for 60 min. A final, 10 min incubation at 95 °C was performed to deactivate the reverse transcriptase. A negative control (−RT) set of samples was also processed, substituting 1.5 μl of nuclease free water for the reverse transcriptase enzyme.

2.5. Primers and PCR amplification

Seven primer sets were utilized to target the following genes: histatin 3 (*HTN3*) and statherin (*STATH*) for saliva, mucin 4 (*MUC4*) for vaginal secretions, matrix metalloproteinase 7 (*MMP7*) for menstrual blood, delta-aminolevulinate synthase 2 (*ALAS2*) for peripheral blood, and protamine 2 (*PRM2*) and transglutaminase 4 (*TGM4*) for semen (Table 1). Singleplex PCR was carried out in duplicate for each cDNA sample and primer set in 25 μl reactions containing the following ingredients: 1.25 μl of 10 mM dNTPs, 2.5 μl of 10× PCR buffer II, 2.5 μl of 25 mM $MgCl_2$, 0.25 μl of 5 U/μl AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1.5 μl of 10 μM forward and reverse primers, and 1 μl of template cDNA, with the final difference made up of nuclease-free water. Each primer set was tested against all 350 stain samples. Positive controls, which consisted of target cDNA template for each primer set, were included in the PCR. Negative controls contained nuclease-free water. The following PCR program was used for all samples: 95 °C for 10 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; a final extension at 72 °C for 5 min, and a 4 °C hold.