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The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids

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ABSTRACT

With current methodology, DNA profiling can identify an individual from a sample of biological material but it does not reveal what body fluid or tissue source the DNA profile originated from. We have developed a multiplex PCR system using messenger RNA (mRNA) that can identify blood, saliva, semen and menstrual blood in individual stains or in mixtures of body fluids. Messenger RNA transcripts specific to each type of body fluid have been identified and a multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) system developed to identify these body fluids along with three housekeeping genes. This multiplex can detect semen and seminal fluid (semen without spermatozoa present). Furthermore, we have targeted the co-isolation of RNA and DNA from the same sample and, with the RT-PCR multiplex, we can determine the type of body fluid present as well as generate a DNA profile(s) from the same stain.

1. Introduction

Biological stains from blood, semen, saliva or mixed body fluids are commonly encountered in forensic casework and there are many case circumstances where it is important to know not only where the sample came from, but also from what body fluid or tissue source the DNA profile originated. The context and relevance of the DNA profiled in the case may depend on the identification of the body fluid(s) from which it has originated.

Current DNA profiling methodology can yield a DNA profile from samples of cellular material that are either too small to see or for which existing screening techniques are inadequate. Such screening techniques can be time consuming, non-specific or lack sensitivity [1–4]. The presumptive tests for blood, such as the commonly used Kastle–Meyer phenolphthalein test, utilise the peroxidase-like activity of haemoglobin. These tests can give false positive results from strong oxidants or from other peroxidases such as those found in plants [1]. There is also the possibility that the stain may test positive for blood, but may be of menstrual origin. Currently there is no reliable method for distinguishing menstrual from circulatory blood. This may have important implications for the case. Saliva stains are difficult to detect and the presumptive tests for salivary amylase have problems associated with the inter- and intra-variability of salivary amylase levels in humans, difficulties in interpreting colour change-based tests, body fluid specificity and poor sensitivity [5–7]. Testing for semen includes the detection of prostate-specific antigen (PSA, also known as p30). PSA is also detectable in male urine [8,9].

A forensic stain may test positive for a particular body fluid, but the DNA profile obtained might come predominantly from some other body tissue e.g. skin cells. Alternatively there could be more than one body fluid or tissue present but the body fluid or tissue that the DNA profile originated from cannot be determined. Developing a method that allows for the identification of the cellular source of the DNA profile is an important step forward for forensic casework. Therefore, a new method for identifying the cellular source of DNA is required for forensic casework.

Messenger RNA (mRNA), once thought to be unstable and to degrade rapidly, has recently been shown to be sufficiently stable to be considered suitable for use in forensic science [10,11]. Profiling of mRNA is now being widely investigated as a new approach to forensic body fluid identification [12–20]. An important pre-requisite for this is the ability to co-extract nuclear DNA and mRNA from the same sample [13,15].

We describe here the development of an mRNA multiplex RT-PCR system that can detect circulatory blood, menstrual blood, saliva and semen (with and without spermatozoa) along with three housekeeping genes, whilst co-isolating nuclear DNA for routine profiling.

2. Methods

2.1. Sample collection

All body fluids were collected from voluntary participants with full informed consent.

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2.2. Human samples

Blood samples were obtained by venipuncture or fingerpricks. Aliquots of blood (50, 20, 10, 5 and 1 μl) and aliquots of saliva (50, 25, 10, 5 and 1 μl) were placed on sterile cotton material or sterile cotton Cultiplast® swabs and the stains left to dry at room temperature overnight. The same procedure was used for freshly ejaculated semen collected in plastic containers (aliquots of 50, 25, 10, 5 and 1 μl), including the collection of semen from vasectomised males. This semen was checked for the absence of spermatozoa by microscopy. Semen was also diluted by 1/10, 1/100 and 1/1000 with phosphate buffered saline and 1 μl samples were applied to sterile swabs. Buccal and menstrual blood (day 2 and 3 of the menstrual cycle) swabs were collected and the swabs left to dry overnight.

Mixed body fluids were obtained by aliquoting volumes of each fluid (from different individuals) in ratios of 1:1, 1:2, 1:9, 2:1 and 9:1 in a total volume of 30 μl for semen/blood and 50 μl for blood/saliva (in duplicate).

2.3. Animal samples

Blood samples from chicken, rabbit, dog, mouse, cat, guinea pig and pig were obtained from the Auckland Forensic Science Service Centre at ESR. Aliquots of blood (50 μl) were placed on sterile cotton Cultiplast® swabs and left to dry overnight. Buccal samples from an Orang-utan were kindly obtained by staff at Auckland Zoo.

2.4. RNA and DNA isolation

Initially total RNA was extracted from blood, menstrual blood, semen and saliva using a modified Juusola and Ballantyne [16] method. Dithiothreitol (0.1 M) was added to the denaturating solution, replacing the 2-mercaptoethanol. Following the addition of sodium acetate, 500 μl of buffered phenol (pH 4) and 100 μl of 1-bromo-3-chloro-propane (Invitrogen) was added, the tubes were mixed by inversion and incubated at 4 °C for 30–60 min until the layers had separated then centrifuged at 16000 × *g* for 20 min.

The RNA was precipitated from the aqueous phase by the addition of 2 μl of Glycoblue (Applied Biosystems) and 500 μl of isopropyl alcohol and incubated overnight at −20 °C and the DNA was precipitated from the interphase and organic phase by the addition of 2 μl of Glycoblue and 500 μl of 100% ethanol and incubated overnight at −20 °C.

Following centrifugation at 16000 × *g* for 20 min (4 °C) the RNA pellets were dried at room temperature for 10 min then resuspended in 25 μl diethyl pyrocarbonate (DEPC) treated water by incubation at 55 °C for 10 min.

The DNA pellets were washed twice with 1 ml of 0.1 M citrate buffer containing 10% ethanol for 30 min to remove residual phenol and centrifugation at 16000 × *g* for 10 min. The DNA pellet was then washed in 75% ethanol for 30 min and centrifuged at 16000 × *g* for 10 min. The DNA was resolubilised in 22.5 μl 8 mM NaOH and left for 60 min at 55 °C before 2.5 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added.

A modified method for the isolation of nucleic acids was used where the total nucleic acids were precipitated from the aqueous phase using 2 μl of Glycoblue and 500 μl 2-propanol as described above. Following the resuspension of the nucleic acids in 25 μl DEPC-treated water, 12 μl was removed for the quantitation and amplification of DNA and the remaining 13 μl was DNase treated and used for the synthesis of cDNA (as described below).

2.5. DNase treatment

The RNA fraction was treated with DNase using TURBO DNA-*free*™ (Ambion®) according to the manufacturer's instructions. The supernatant was removed and placed into a clean RNase-free tube and either used immediately or stored at −20 °C until required.

2.6. RNA and DNA concentration determination

The concentration of RNA from the samples and the control RNA were determined using an Agilent 2100 Bioanalyzer with the use of the RNA 6000 pico kit. The manufacturer's instructions were followed and 1 μl of total RNA extract was used.

The DNA concentration was measured using the Quantifiler™ system (Applied Biosystems), using an ABI 7500 real-time PCR machine. A total of 2 μl of DNA was used in a 25 μl reaction, according to the manufacturer's instructions.

2.7. cDNA synthesis

The cDNA was synthesised in 20 μl reactions using Superscript III® (Invitrogen™) reverse transcriptase, 6 μl of the extracted RNA and 1 μl (50 ng) random hexamer primers. Negative controls were included for each RNA sample. The cDNA was either used immediately or stored at −20 °C until required.

Control human RNA (tissue origin unknown, Applied Biosystems) was used for sensitivity studies, with a range of concentrations (0.05–2.5 ng RNA) used to synthesise cDNA and for subsequent use in the mRNA multiplex assay.

2.8. Body fluid genes

The genes chosen for identifying specific body fluids were identified by a literature search and from previous studies [10–20] and are briefly summarised here: Glycophorin A (GlycoA) for blood, matrix metalloproteinase 11 (MMP11) for menstrual blood, histatin 3 (HIS) and statherin (STATH) for saliva, protamine 2 (PRM2) for spermatozoa and transglutaminase 4 (TGM4) for seminal fluid. Three housekeeping genes were also included in the multiplex; transcription elongation factor 1α (TEF), glucose 6-phosphate dehydrogenase (G6PDH) and ubiquitin conjugating enzyme (UCE). Table 1 lists the primer sequences used in the multiplex assay. All primers were designed using Primer Express V3.0 Software (Applied Biosystems). The primers were designed to span intron/exon boundaries where possible, with the exception of PRM2 due to the size of this gene and the position of the only intron within this gene.

2.9. cDNA multiplex polymerase chain reaction

A volume of 2 μl of cDNA (from the 20 μl cDNA reaction) was amplified in a total reaction volume of 25 μl. The Qiagen multiplex PCR Kit was used for the multiplex PCR assay. The reaction mix contained 12.5 μl of reaction buffer, 2.5 μl of 10× primer stock and 8 μl sterile water. The final optimised concentrations of primers (forward and reverse) in the multiplex reaction were: GlycoA 0.2 μM, MMP11 0.1 μM, HIS 0.25 μM, STATH 0.25 μM, PRM2 0.05 μM, TGM4 0.1 μM, TEF 0.025 μM, G6PDH 0.2 μM and UCE 0.125 μM. The PCR conditions were 95 °C for 15 min, 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s for a total of 30 cycles and a final step of 72 °C for 45 min.

Amplification products were run on 3130 genetic analyzers (Applied Biosystems) using GS-500 size standards and analysed using GeneScan V3.7 (Applied Biosystems).

2.10. DNA amplification

The co-extracted DNA was amplified using the SGM + AmpF*l*STR system (Applied Biosystems) and analysed using