Successful Identification of Two Years Old Menstrual Bloodstain by Using MMP-11 Shorter Amplicons

Sir,

Recently mRNA amplification of biological stains has become a convincing instrument for body fluid identification in forensic casework (1). mRNA recovery in sufficient quantity and quality for RT-PCR analysis and its stability in biological stains up to 15 years were demonstrated by Bauer et al. (2). These authors developed a powerful protocol to detect endometrial cells on dried menstrual bloodstains via RT-PCR of several markers among which metalloproteinase 11 (MMP-11) was found to be sensitive and tissue-specific (3,4). This protocol was successfully applied in our experimental studies on laboratory prepared menstrual bloodstains aged up to 1 year to amplify MMP11 and Er-α receptor gene (data not shown), but in a two years old sample, consisting of knickers with a presumed large spread menstrual bloodstain mixed with urine and stored dried at room temperature, the results were not reproducible and in multiple assays, and although the housekeeping gene was positive, MMP-11 amplification failed even to increase the size of the sample. Despite the interpretation of MMP-11 negative results (4), we modified the original protocol consisting of 55 cycles amplification for a 455 bp PCR product to achieve a more sensitive detection approach.

RNA isolation was performed using a fast method that allows recovery of RNA from 200 bp. For reverse transcriptase (RT) reaction, the random primers technique was preferred since the RNA degradation is significantly correlated with the storage interval and bacterial overgrowth that causes mRNA further fragmentation. Under these conditions, 3' poly (A) tails become dissociated from the rest of the molecule, and the oligo-dT primers, which bind to the poly (A) tail, would be ineffective, whereas the random primers would still to able to anneal and generate a product (1).

For amplification we selected the primer set and the PCR conditions described in a recent study on endometrial biopsy samples (5). The housekeeping gene L19 of 169 bp in size (6) was used as positive control in all stain samples submitted to the analysis. Total RNA was extracted with RNeasy Mini Kit (QIAGEN, Hilgen, Germany) from 0.5–1 cm^2 stain samples, and DNase digestion was performed by DNase 1 (Invitrogen, Netherland) following manufacturer's instructions. RT reaction was carried out with random primers using First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. A volume of 2–5 µL of the RT reaction was amplified in a total reaction volume of 25 µL. The reaction mixture contained 1X PCR buffer, 1.5 mM MgCl$_2$, 0.25 mM each dNTPs, 0.4 µM of each primer (MMP-11 forward 5'-ATTTGGTTCTTCCA-AGGTGCTCAGT-3'; MMP-11 reverse: 5'-CCTCGGAAGAA-GTAGATCTTGTTCT-3'), and 1.5 units AmplitTaq DNA polymerase (AmpliTaq, Perkin Elmer Roche, USA) using a thermocycler manufactured by Biometra (Biometra, Gottingen, Germany). PCR conditions consisted of 30 s at 94°C, 1 min at 63°C, and 1 min at 72°C for 30–35 cycles to amplify a product 155 bp long. RT-PCR products were resolved on 2% agarose gels stained with ethidium bromide. Positive results for MMP-11 were obtained in 90% of assays in different areas of the stain with L19 housekeeping gene detected in all the samples. Possible false positive results deriving from genital tract tumor, genital infections, or from the placenta could be excluded by medical anamnesis and gynacological examination as suggested by Bauer et al. (4).

The significative increase in the success rate of MMP-11 amplification appears to be linked to the reduction in size of amplicons. mRNA fragmentation could determine a loss of the molecule for PCR mainly for longer products that are also kinetically disfavored in amplification, so a 455 bp long product appears disadvantaged. Nevertheless since DNase treatment is ineffective in extinguishing all genomic DNA signal with incomplete degradation (7), 55 cycles of amplification exposes to genomic DNA amplification risk, especially for processed pseudogenes indistinguishable from mRNA or to artifact products (8). We think that the described approach represents an advance that may resolve the potential problems raising from old and degraded samples and from extreme PCR conditions.

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