TECHNICAL NOTE

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Detection of Epithelial Cells in Dried Blood Stains by Reverse Transcriptase-Polymerase Chain Reaction*

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ABSTRACT: The identification of menstrual blood stains can be improved by detection of messenger ribonucleic acid (mRNA) specific for epithelial (endometrial) cells. RNA molecules, however, are believed to be unstable and require careful sample processing. In this study, we have investigated the extraction of RNA suitable for reverse transcriptase-polymerase chain reaction (RT-PCR) from dried blood stains stored for up to six months. With a modified RNA isolation protocol, it was possible to obtain RNA from dried blood stains with at least 5×10^2 leukocytes. In an additional experiment, we evaluated the RNA isolation from mixed stains composed of leukocytes and T47D cells, a breast cancer-derived cell line with epithelial origin. Detection of 10^2 T47D cells in a total number of 10^5 leukocytes was possible by amplification of cytokeratin 19 mRNA and progesterone receptor-mRNA specific for hormonally regulated epithelial cells. In both experiments amplification results were not dependent on storage time with similar data from one day to six months. Furthermore, it was possible to identify dried menstrual blood samples by showing the presence of mRNA specific for epithelial cells. These results demonstrate for the first time, that RNA suitable for RT-PCR, can be isolated from forensic specimens stored up to at least six months, and that a small number of epithelial (endometrial) cells can be identified in dried blood specimens. Using this method, it will be possible to identify the origin of small and partially degraded blood samples which can be especially useful in forensic evaluation of cases with sexual offense.

KEYWORDS: forensic science, RNA stability, RT-PCR, GAPDH, cytokeratin 19, glyceraldehyde-3-phosphate dehydrogenase, progesterone-receptor, T47D cells, menstrual blood, dried blood stains

In the forensic examination of blood stains, the identification of the source of bleeding is frequently of crucial importance. Menstrual blood, for example, must be distinguished from traumatic bleeding, especially in cases of sexual assault.

A usual method of identifying menstrual blood stains is the microscopic detection of endometrial cells. Other methods are based on specific protein patterns of menstrual blood samples (1,2). However, these methods require sufficient quantities of blood and are sensitive against prolonged and inadequate storage. Moreover, if only stains of limited size are available, simultaneous DNA analysis often is not possible.

The detection of messenger ribonucleic acids (mRNA) in dried blood stains may be a possibility to overcome this problem. In the cell, mRNA molecules are responsible for the transfer of genetic information from DNA to proteins. Therefore, the mRNA expression pattern is dependent on cell type and cell function and provides cell-specific informations, similar to proteins, on the other hand, mRNA is accessible to the same molecular analysis techniques as DNA due to their common nucleic acid structure. The method is based on the assumption that endometrial cells, which are constantly present in menstrual blood, express a specific mRNA pattern that makes them distinguishable from leukocytes and other epithelial cells. The development of reverse transcriptase polymerase chain reaction (RT-PCR) allows, in contrast to Northern blotting, the analysis of small and even partially degraded RNA samples. The isolation protocols for RNA and for DNA are only slightly different due to the similar molecular composition. DNA isolation, therefore, can be performed during the same laboratory procedure with only a few additional steps.

However, RNA molecules are frequently believed to be unstable and to undergo rapid degradation in the living as well as in the dead cell due to the action of ribonucleases (RNAses) present in nearly all biological materials. On the other hand, it has been demonstrated that RNA suitable for RT-PCR can be obtained from various human tissues like brain (3) or liver (4) up to several days after death. In other experiments, RNA could be isolated from blood spots dried on filter paper and stored up to several years (5,6). RNA molecules, therefore, seem to be, under certain conditions, more stable then generally expected.

In order to establish RNA analysis as a tool in identifying menstrual bleedings as source of dried blood stains our objective was to show that RNA suitable for RT-PCR can be isolated from small dried blood stains stored for different time intervals and that a relatively small number of epithelial cells can be identified in mixed stains. Furthermore, we report a first and preliminary application of this method to menstrual blood stains.

Materials and Methods

Preparation of Samples

Blood samples from several healthy donors were collected in EDTA-coated tubes. After leukocyte counting aliquots of the sam-

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ples containing 1 to 10^4 cells, respectively, were placed on a clean cotton weave and air-dried. An equivalent piece of the same cotton weave without blood was used as negative control.

T47D cells were a gift of Dr. Hermann Kneitz (Institute of Pathology, University of Wuerzburg). Briefly, they were cultured in RPMI-1640 medium (Sigma) with 10% fetal calf serum (FCS) and allowed to grow for five days in a humidified CO_2 (5%) incubator at 37°C. Blood samples were taken as described above. The T47D cells were simultaneously harvested with trypsin, counted and resuspended in RPMI without FCS. For the dilution experiment, aliquots of the cell samples containing 1 to 10^5 cells were mixed with 10^5 leukocytes from the previously taken blood to a final volume of 25 μL respectively, placed onto a clean cotton weave and allowed to air-dry. Controls enclosed 25-μL blood without T47D cells and T47D cells suspension containing 1 to 10^4 cells without leukocytes. Samples of menstrual blood were obtained on customary tampons from several donors and air-dried. Control samples were taken by finger pulp micropuncture. All samples were stored at room temperature.

Isolation of RNA

All reagants were prepared with water treated with diethyl pyrocarbonate (DEPC) and autoclaved to prevent ribonuclease activity. Glassware and instruments were rinsed with DEPC-treated water and autoclaved. After one and 28 days and six months, the blood stains were cut out and placed directly into 100 μL denaturing solution containing 4 M guanidiniumisothiocyanate, 25 mM sodium citrate pH 7, 0.5% N-lauroylsarcosine, and 0.1 M 2-mercaptoethanol at 56°C for 30 min. Another assay consisted of placing the stains in 100 μL water treated with (DEPC) for at least 30 min with subsequent pelleting of cells prior to adding 100 μL denaturing solution. The cotton weave was removed by centrifugation and the isolation procedure performed according to the single-step-method (7). The mixed stains were placed into 800 μL of denaturing solution and submitted to the same isolation procedure. The final pellet in both experiments was redissolved in 10.5 μL DEPC-treated water. The RNA purity and concentration was determined by measuring the optical density at 260 nm.

Isolation of mRNA

Isolation of mRNA was performed using the QuickPrep Micro mRNA Purification Kit from Pharmacia Biotech. Briefly the blood stains were placed into 0.4 mL of extraction buffer and heated to 56°C for 30 min. After mRNA isolation according to the manufacturers instruction the eluted samples were precipitated adding 10 μL of glycogen solution, 40 μL of potassium acetate solution and 1 mL of 95% ethanol. The resulting invisible pellet was redissolved in 10.5 μL of DEPC-treated water.

Reverse Transcription

The RT-Mix (0,5 μg Oligo-dT-primer, DTT, buffer, NTPs) was added to the RNA-solution to a total volume of 18,5 μL. To remove possible contamination with genomic DNA digestion with deoxyribonuclease I (DNAse I, Pharmacia) was carried out in the RT-Mix prior to reverse transcription (1 U, 30 min at 37°C). DNAse was inactivated by heating to 75°C for 10 min. Subsequently, reverse transcriptase (Superscript II, Gibco, 200 U) was added and the reaction performed according to the manufacturer's instructions.

PCR

2 μL of the RT-solution were amplified in a total reaction volume of 50 μL containing 1,5 mM MgCl and 2 U TAQ-Polymerase (AmpliTaq, for PR-primers: AmpliTAQ Gold, Perkin Elmer). The primers had the following base composition: Glyceraldehyd 3 phosphate dehydrogenase (GAPDH) forward GTGGAAGGACTCATGACCACAGTC; reverse GTGGTGGACCTCATGGCCCACATG; Cytokeratin 19 (CK 19) forward CAAGATCCTGAGTGACATGCG; reverse AGCTCAATCTCAAGACCCTG; Progesterone receptor (PR) forward CAGTGGGCAGATGCTGTATTTTGC; reverse CTTGAAGCTTGACAAACTCCTGTGG; VWA and FES/FPS as described elsewhere (8)

PCR conditions consisted of a denaturing step (94°C, 4 min) followed by 35 cycles (94°C, 1 min; 60°C, 1 min; 72°C 1 min) and a final extension step (72°C, 5 min) for GAPDH and CK 19. For PR-specific PCR, a 9 min 94°C pre-PCR heat step was performed followed by 48 cycles (94°C 1 min, 62°C 30 sec). The PCR-conditions for VWA and FES/FPS were described previously (8). The reactions were performed on thermocyclers manufactured by Biometra and by Perkin Elmer (hot start PCR). The amplification products were visualized on 2%-agarose gels stained with ethidium bromide.

Sequencing

PCR products amplified with GAPDH-specific primers were purified with Centricon-100 columns. The DNA concentration was measured spectrophotometrically. Between 40 and 80 ng DNA were used with the Dye Terminator Cycle Sequencing kit (ABI-Perkin-Elmer) according to the manufacturer's instructions. Extension products were purified by ethanol precipitation using 3M sodium acetate, pH 4.6, and 95% ethanol. Data were collected on an Applied Biosystems 310 sequencer and analyzed using ABI programs Sequence analysis version 2.1.1 and Sequence navigator version 1.0.1.

Results

RNA-Isolation from Blood Stains

RNA concentrations of all samples were to low to be measured by spectrophotometry. After RT-PCR, GAPDH-Signals were constantly obtained from all blood stains containing 5×10^2 cells and more using the modified single-step method with direct rehydration of stains in the guanidiniumisothiocyanate-containing solution (Fig. 1). The stains with 10^2 and 5×10^1 cells showed variable amplification results. No significant differences could be detected at the different time points with similar amplification results from one day to six months after sample preparation. DNA-specific PCR with VWA and FES-Primers was always negative. Sequencing of

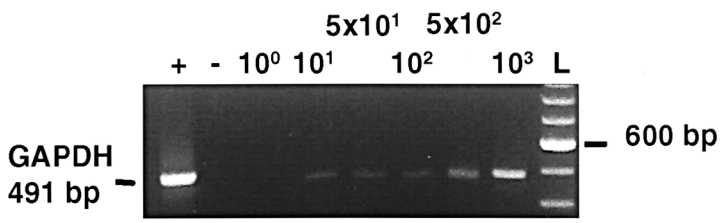

FIG. 1—*RT-PCR from RNA isolated from dried blood stains stored for 4 weeks with GAPDH-specific primers. L = 100 bp-ladder, the respective leukocyte number is indicated above the lanes.*

the PCR-products showed complete accordance with the expected base sequence. Neither direct mRNA-isolation from the stains nor mRNA-isolation from previously isolated total RNA provided constant amplification products after RT-PCR. Rehydrating the stains in water lead to inconsistent results with frequent lack of any amplification signal.

RNA-Isolation from Mixed Blood Stains

The total amount of RNA isolated from the stains containing 10^5 leukocytes was maximally 250 ng. In mixed stains with 10^4 T47D cells up to 340 ng RNA was obtained by the modified single step method. In all samples, RT-PCR with GAPDH-primers produced a constant signal. The amplification product for CK 19 showed declining intensity with a detection limit between 10^2 and 10^3 T47D cells, for PR with hot-start-PCR again a limit between 10^2 and 10^3 cells (Fig. 2). Again rehydration of stains in DEPC-treated water frequently lead to negative results or weaker bands. Genomic DNA was absent as proved by PCR analysis of short tandem repeat sequences. No influence of the storage time interval on the amplification efficacy could be detected.

Menstrual Blood Samples

RNA isolation with positive GAPDH signals was possible from all samples. Amplification products for CK 19 and PR were obtained from all but one sample (Fig. 3). Blood stains obtained by micropuncture were negative for CK 19 and PR.

Discussion

For RNA analysis, it is necessary to use a reliable method which proves the presence and integrity of RNA. Northern blotting requires sufficient concentrations of RNA usually not provided by forensic samples. The grade of RNA degradation is frequently shown by the presence of two bands for ribosomal RNA (18S and 28S) in an agarose gel run under denaturing conditions. Again, this method does not work with small RNA amounts isolated from blood stains. Thus, like in forensic DNA analysis, PCR methods with previous synthesis of complementary DNA (cDNA) by reverse transcription (RT-PCR) have to be used. Since PCR cannot differentiate between genomic DNA and cDNA, it is necessary to choose conditions for reverse transcription and PCR that exclude the presence of genomic DNA and assure the transcription and amplification of RNA. This can be done by demonstrating the presence of constantly and ubiquitously expressed mRNAs transcribed from so-called housekeeping genes (9). For our studies, we chose glyceraldehyd-3-phosphate dehydrogenase mRNA (GAPDH), which is one of the best known and most intensively examined housekeeping genes with constant transcription level (10). The presence of an amplification product of the expected size with GAPDH-specific primers and the confirmation by sequencing, therefore, proves the presence of mRNA in the original sample if DNA contamination can be excluded. Under usual conditions, the modified single-step method (7) should provide virtually pure RNA solutions free of DNA but, in practice, contamination never can be totally avoided. For this reason, the primer binding sites in our experiments were placed on different exons so that genomic DNA amplification would have lead to an additional amplification product of different length. The GAPDH forward primer was designed to span an exon-exon junction restricting the primer binding site to the processed mRNA respectively reverse-transcribed cDNA (Fig. 4). This can prevent amplification of the corresponding gene sequence but not of retropseudogenes which are known to exist for GAPDH (10) as well as for cytokeratin 19 (11). Therefore, we used digestion with DNAse prior to reverse transcription. Since an additional phenol-chloroform extraction with precipitation to remove DNAse led to intolerable loss of RNA, the enzyme was inactivated by heating up to 75°C for 10 min as described by Huang et al. (12). This highly efficient method was simultaneously used for RNA denaturing in order to diminish pipetting steps since all reagents for reverse transcription except the reverse transcriptase were already present in the reaction mixture.

The complete absence of DNA after digestion was confirmed by GAPDH-primed PCR without reverse transcription and by PCR with DNA-specific primers (STR-primer: FES and VWA), which are routinely used in forensic practice (13). The amplification products obtained by our experiments, therefore, prove the successful isolation and transcription of RNA from dried blood stains.

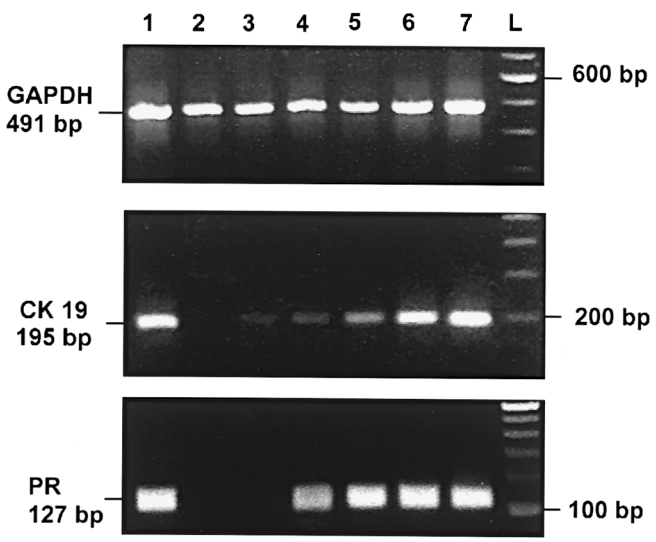

FIG. 2—*RT-PCR from RNA isolated from mixed blood stains stored for 4 weeks with primers specific for GAPDH, CK 19 and PR. L = 100 bp-ladder, Lane 1 = T47D-cells, 2-7 mixed stains: Lane 2: 10° T47D-cells; 3: 10^1; 4: 10^2; 5: 10^3; 6: 10^4; 7: 10^5.*

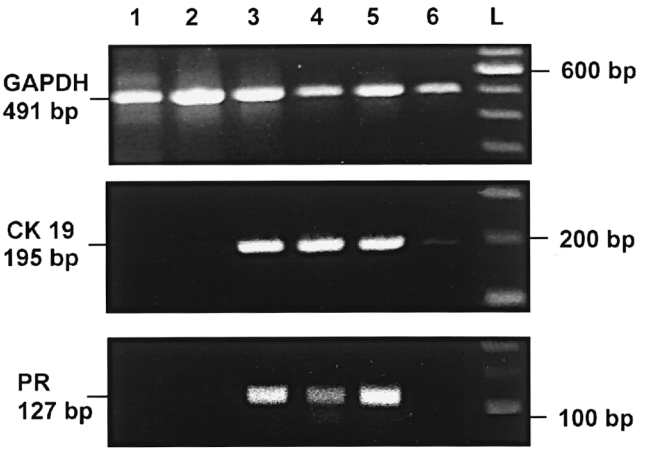

FIG. 3—*RT-PCR from RNA isolated from dried menstrual blood samples (Lanes 3-6) and from blood taken by micropuncture (Lanes 1&2). Primers as described in Fig. 2.*

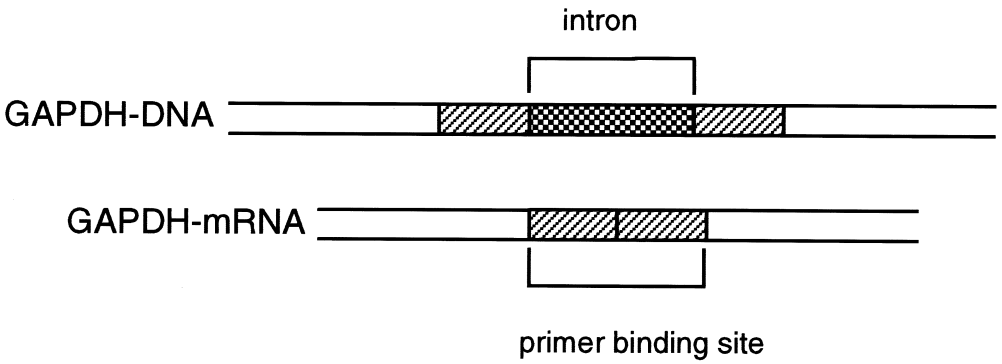

FIG. 4—*Schematic position of the GAPDH forward primer binding site.*

The RNA is of high integrity, since the GAPDH-product is composed of 491 base pairs and the distance between the 3′-end of the reverse primer binding site and the poly-A-tail of the GAPDH-mRNA spans another 225 base pairs, thus indicating a length of at least 716 bp for the GAPDH-mRNA isolated from the dried blood stains.

Although there are a lot of commercially available protocols and kits for RNA isolation, we selected the traditional single step method first described by Chomczynski and Sacchi (7) since it offered the highest variability and the possibility to recover DNA suitable for PCR from the organic phase and interphase (data not shown). The method had to be adjusted to the small number of cells available for isolation and to the need for previous sample rehydration. The most convincing results were obtained by placing the stains directly into heated denaturing solution, which in one step rehydrates the sample, breaks up the cells, and prevents ribonuclease activity. Other possibilities like previous rehydration in DEPC-treated water lead to inconsistent results probably due to RNA degradation during rehydration. Direct mRNA-isolation with poly-A-coated affinity columns was not successful probably because the relatively high elution volume (200 μL) resulted in a highly diluted mRNA solution which had to be precipitated again.

So far, our results confirm that RNA is stable enough to be extracted from dried and stored blood stains and to be analyzed by RT-PCR. This is in accordance with reports using filter paper as carrier for dried blood spots (5,6). In experiments studying HIV-1 RNA levels (14), or fragile X syndrome (15) dried blood or plasma spots were successfully used for clinical investigations. In contrast to our experiments these studies, however, did not or not sufficiently consider possible false positive results by amplification of genomic DNA or pseudogenes which is of crucial importance in forensic science. Furthermore, the key question is not the analysis of gene transcripts present in every cell but rather the identification of a small number of a specific cell type among a large majority of blood cells.

To investigate this question, we used an experimental design analogous to a model which is currently widely tested in clinical medicine to detect circulating carcinoma cells in blood samples (16,17).

For these experiments, variable numbers of T47D cells, a well established breast cancer-derived cell line, which express epithelial cell markers not regularly found in blood cells, were mixed with blood containing a constant number of leukocytes. Epithelial cells possess specific cytoskeleton proteins, the cytokeratins (18), which can be used to prove the epithelial origin of cells at the protein or mRNA level. Among the cytokeratin family cytokeratin 19 is constantly expressed in T47D cells. Although a so-called illegal transcription in blood cells detected by nested PCR was recently published (19), we could not observe any CK-19 expression in our experimental controls (dried blood stains without addition of T47D cells or directly isolated leukocyte RNA). In addition, the ethidium bromide staining intensity for the CK 19 amplification product was strongly correlated with the T47D cell number (Fig. 2) suggesting that CK 19 mRNA was derived exclusively from T47D cells.

Our data show that as low as 10^2 T47D, cells could be detected by CK19-specific RT-PCR which corresponds to a detection ratio of 1 T47D cell among 10^3 leukocytes. Referring to forensic practice, this is equivalent with the identification of 1 epithelial cell in 0,2 μL blood. Further differentiation between epithelial cells of different origin has to rely on organ- or cell type-specific markers. T47D cells, for example, express progesterone receptor mRNA, which is only found in hormonally regulated tissues as endometrium, but not in other epithelial cells. As expected, the PR mRNA expression level proved to be lower than the CK 19 level and required more sensitive hot-start PCR to become detectable. The detection limit could be reduced to 10^2 cells with ethidium bromide staining in agarose gels. More sensitive detection methods probably will allow to further lower this limit and are currently under investigation.

To show that our experimental results can be of diagnostic value in forensic medicine, we applied the method described above to several menstrual blood samples. It was possible to identify epithelial cells in these samples by RT-PCR specific for CK 19 and PR. In blood stains obtained by micropuncture which is comparable to traumatic injury, no epithelial cells could be detected. Since in practice, it cannot be excluded that traumatic bleedings contain enough epithelial cells to produce specific PCR signals and since at least CK 19 is expressed in many epithelial cells, further research has to identify mRNA markers specific for endometrium during menstruation. A panel of primer pairs is currently tested on different RNA samples (endometrium, vaginal mucosa, and skin) for their ability to discriminate the sample origins.

In conclusion, we have developed a method to extract RNA suitable for RT-PCR from blood stains dried on cotton weave containing only 10^2 cells. False positive results due to amplification of genomic DNA or pseudogenes could be excluded. Obviously, air-drying of blood is an equally effective fixation method for RNA as for DNA. Once dried, no further significant RNA degradation occurs at least for a time period of 6 months. Limiting factors may be the same as for DNA (insufficient drying, bacterial overgrowth). Us-

ing this method, it was possible to identify epithelial cells in artificial blood stains as well as in dried menstrual blood samples by showing the presence of mRNA common to all epithelial cells (cytokeratin 19) and specific for hormonally regulated epithelial cells (progesterone receptor). With this background, further research will focus on searching for mRNA markers specific for endometrial cells.

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