The effect of temperature on age estimation of semen stains on porous versus non-porous surfaces using messenger ribonucleic acid measurement

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Abstract

Background: While mRNA can be used to identify the type of a body fluid, its degradation can also give some indication of the time interval since it was deposited. This study was conducted to evaluate the effect of temperature on the age estimation of human semen stains using mRNA deposited on porous versus non-porous surfaces at different time intervals. **Methods:** Ten semen samples were applied on two different media (glass and cotton) and exposed to three different temperatures (4°C, room temperature, 40°C) and examined at three time intervals (0, 45, and 90 days). The semen-specific mRNA markers PRM1 and PRM2 were quantitatively assessed along with a reference gene, beta-actin, using reverse transcription-quantitative polymerase chain reaction. **Results:** Mean Cq values of mRNA markers (PRM1 and PRM2) and the reference gene (beta-actin) increased with time of storage at different temperatures on both examined media. The mean quantification cycle (Cq) values of PRM2 were lower than PRM1, indicating that the levels of PRM2 marker in semen stain were higher than those of PRM1 marker. However, the mean Cq values of PRM2 at each time interval were not significantly different between temperatures, while PRM1 showed statistically significant differences in mean Cq values between temperatures at day 45 on both media. **Conclusion:** These results indicate that PRM2 can serve as a reliable mRNA marker to estimate the time since deposition of semen stain at different temperatures on two different media.

Keywords: Semen age, PRM1, PRM2, temperatures, media, mRNA, RT-qPCR.

Introduction:

Some RNA forms such as messenger (m)RNA can disclose the activities of various genes and the identity of their respective cells and tissues. Also, RNA may be used to recognize the body fluid type, while the mechanism of RNA degradation can serve as a useful indicator of the time interval since it was deposited **(1)**.

The time passed since deposition is very critical information for criminal investigations. Knowing the time when body fluid was deposited at a crime scene can help investigators determine when a crime occurred. Additionally, deposition of samples which does not match the time when the crime is expected to have occurred may be ignored **(2)**.

Different body fluids contain nucleic acids which interact with their surroundings. The surrounding atmosphere affects body fluids both by preserving the samples and causing degradation, which is major problem for investigators. Degradation passively influences the nucleic acid containing all the information which can help solving a crime **(3)**.

Semen is the most trustworthy marker in rape, sodomy, and different forensic cases. It can be used to prove sexual assault and identify suspects. Also, it serves as an indicator of the time when a crime occurred **(4)**.

Methodology

Study design and setting

This research was a prospective analytical study. It was performed at the Faculty of Medicine of Cairo University, Egypt, during the period between November 2020 and February 2021 in collaboration with the Department of Medical Biochemistry and Department of Andrology Faculty of Medicine of Cairo University. The study was approved by the Ethical Committee of Forensic Medicine and Clinical Toxicology Department and the Ethical Committee of Faculty of Medicine, Cairo University.

Study population

Participants

A total of 10 semen samples with normal quantity and quality were collected from healthy males with an age range of 20 to 60 years, after obtaining their informed consent. Individuals with azoospermia, severe oligozoospermia, congenital abnormalities such as undescended testes, and chronic diseases such as diabetes mellitus were excluded.

Study measurements

The human semen samples were directly deposited in sterile containers and each divided into two equal portions:

a) The first portion, 50 μL of semen spotted onto nine pieces of sterilized white cotton cloth (5 × 10 cm) as an example of porous surface. Three of these nine samples were stored at room temperature in a dark dry area to simulate natural aging, three samples were stored in the refrigerator at 4°C, and the remaining three samples were stored in an incubator at 40°C.

b) The second portion, 50 μL of each semen sample spotted onto nine pieces of sterilized glass slides (5 × 10 cm) as an example of non-porous surface. Three of these nine samples were stored at room temperature in a dark dry area to simulate natural aging, three samples were stored in the refrigerator at 4°C, and the remaining three samples were stored in an incubator at 40°C.

While the semen samples were collected in November 2020, the room temperature during the study was ranging from 14°C–25°C.

The mRNA markers PRM1 and PRM2 and the reference gene beta-actin were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at the different time intervals of 0, 45, and 90 days.

RNA extraction and purification

The RNA extraction and purification procedures were conducted according to the instructions provided with the nucleic acid extraction kit (NucleoSpin®), while RNA concentration was measured using a Beckman dual spectrophotometer at a 260–280 nm ultraviolet invisible wavelength.

Reverse transcription and RT-qPCR

The RT-qPCR assays were conducted with a ViPrime One Step RT-qPCR 2× SyGreen Mix (HRox, cat. no QR8602-100, Malaysia) using a StepOne Real-Time PCR Applied Biosystems detection system operating version 3.1 of the software (StepOne™, USA). The study was done by using 5 μL of total RNA and 1 μL of the gene-specific primer. The thermal cycler settings were as follows: 1 cycle of reverse transcription for 10 minutes at 55°C, an initial activation for two minutes at 95°C, 40 cycles of denaturation at 95°C for five seconds, then annealing and extension at 60°C for one minute. Beta-actin was selected as the reference gene considering that it is an important housekeeping gene showing constant transcription levels in response to experimental manipulation in most tissues. The primer sequences for the measured genes were as follows:

PRM1:
Forward primer: 5'-ATGGCCAGGTACAGATGCTGTCGCAG-3'
Reverse primer: 5'-GTACCTGGGGCGGCAGCACCTCATGG-3'

PRM2:
Forward primer: 5'-GCTGAGCCCGGAGCACGTCGAGGTC-3'
Reverse primer: 5'-AGGGGGTCACCTAGGGACTCTCTGC-3'

Beta-Actin (reference gene):
Forward primer: 5′-TGTTGTCCCTGTATGCCTCT-3′
Reverse primer: 5′-TAATGTCACGCACGATTTCC-3′

Expression values of the studied markers

After the RT-qPCR run, the data were expressed in quantification cycle (Cq). Cq values are opposite to the levels of target nucleic acids present in the samples. Lower Cq values indicate higher levels of the target nucleic acid. Higher Cq values represent lower levels of the target nucleic acid.

Statistical analysis

As previously conducted **(5) & (6),** data were analyzed using the statistical software SPSS, version 26. To compile the data for quantitative variables and frequencies, the mean and standard deviation were employed. When comparing two groups, an unpaired Student's t-test was used. With multiple comparisons, an analysis of variance was employed, followed by a post-hoc test. Pearson correlation coefficients were used to calculate correlations between quantitative variables. *P* values under 0.05 were considered as statistically significant.

Results

On the glass media, comparison between the different markers at each time interval examined showed that the mean Cq values of the mRNA markers PRM1 and PRM2 and reference gene beta-actin were significantly increased with the time of storage reaching highest levels at day 90 ($p < 0.001$). This increase was observed at the different storage temperatures (room temperature, 4°C and 40°C) **(Table 1)**.

Comparing the mean Cq values of each marker at different temperatures on the glass media showed that the mean Cq values for PRM1 and beta-actin were significantly different at day 45 ($p < 0.05$) **(Table 2)**. The Cq value was higher at room temperature at 40°C *versus* 4°C for PRM1 and beta-actin **(Table 3)**.

On the cotton media, comparison between different markers at each time interval showed that the mean Cq values of the mRNA markers PRM1 and PRM2 and the reference gene beta-actin were significantly increased with the time of storage, reaching highest levels at day 90 ($p < 0.001$). This increase was observed at different storage temperatures (room temperature, 4°C and 40°C) **(Table 4)**.

Comparison of the mean Cq values of each marker at different temperatures on cotton media showed that the only marker presenting statistically significant difference in its mean Cq values was PRM1 at day 45 ($p < 0.05$) **(Table 5)**. Its mean Cq value was higher at room temperature than at 4°C **(Table 6)**.

Comparing the mean Cq values of all markers on both media (glass and cotton) at each time interval at room temperature revealed that the mean Cq value of PRM1 was significantly higher on cotton than glass media at day 0, while beta-actin levels were significantly higher on glass media ($p < 0.05$). Otherwise, there was no significant difference between media in the mean Cq of any marker at any time interval **(Table 7)**.

At 4°C, there was a statistically significant difference between glass media and cotton media in the mean Cq value of PRM1 at days 0 and 45 (with higher values on cotton media) ($p < 0.05$) Besides, there was no statistically significant difference between media in mean Cq of any marker at any time interval **(Table 8)**.

Comparing the mean Cq value of all markers on both media (glass and cotton) at each time interval at 40°C also indicated that there was no statistically significant difference between media in mean Cq value of any marker at any time interval **(Table 9)**.

Discussion

Biological evidence collected from crime scenes can be affected by a variety of environmental conditions, while the surroundings are expected to change the kinetics of RNA degradation **(7)**.

The objective of this study was to evaluate the effect of temperature on the estimation of the time since deposition of semen on porous surface (cotton) *versus* non-porous surface (glass). The semen-specific mRNA markers PRM1 and PRM2 were assessed along with a reference gene (beta-actin) at three time intervals (0, 45, and 90 days) and three different temperatures (room temperature, 4°C and 40°C).

Cq values are opposite to the levels of target nucleic acid contained in the study sample. Lower **Cq** values indicate high levels of the target sequence. Higher **Cq** values mean lower levels of the target nucleic acid **(8)**.

In the current study, the mean Cq values of both semen-specific markers (PRM1 and PRM2) and the reference gene (beta-actin) increased with increasing time of storage of specimen, reaching the highest level at day 90 at the three temperatures and on both media. These came in agreement with **(9)** who studied the degradation pattern of mRNA of semen, saliva, nasal discharge, and vaginal fluid in samples applied on cotton media and stored for one year at different temperatures, using RT-qPCR as a quantification technique. Their study showed a gradual increase in Ct values of the studied markers across storage times. Similar results were observed by **(10)** in another study on blood specific mRNA markers examining the degradation rate of blood specific mRNA markers (HBA, HBB and PBGD) to determine the age of blood stains. Their results showed that different mRNA markers degraded with different rates over time. Another study conducted by **(11)** on three types of body fluids (semen, blood and saliva), stored on nuclease-free collection cards, indicated that the decay of transcripts from housekeeping genes like GAPDH, ACTB, and B2M can be studied to estimate sample age. This study concluded that there is a global decrease in mRNA abundance in aging stains.

These results were in accordance with **(12)** who studied the applicability of using RNA degradation as a method for detecting age of stored blood samples using Cq values of mRNA markers. Their study revealed that **ΔCq** values of dried blood samples were increasing with time and could be used as a method for identifying the age of dried blood stains.

When comparing the three different temperatures at day 45, we found that the mean Cq value of PRM1 at room temperature was significantly higher than its mean Cq value at 4°C on both media ($P < 0.05$), while its mean Cq value at 40°C was significantly higher ($P < 0.05$) compared to its mean Cq value at 4°C, but on glass media only. Beta-actin also showed a statistically significant increase in its mean Cq value at room temperature compared to 4°C on glass media ($P < 0.05$). These results were consistent with **(7)** who studied RNA degradation in aged blood stains at three different temperatures (4°C, 20°C and 37°C) and revealed that the mean Cq values of RNA transcripts raised with increasing temperatures.

Also **(13)** investigated the effects of heat and humidity on viral RNA degradation. The authors concluded that both heat and humidity can increase the rate of viral RNA degradation, while the effect of heat was more pronounced.

These results were not in accordance with **(9)** who concluded that Ct values of studied markers in aged samples were higher at room temperature compared to 35°C and explained this finding by an effect of humidity and light at room temperature on RNA degradation.

According to the study, there was no statistically significant difference in the mean Cq values of PRM1 and beta-actin between the examined temperatures at days 0 and 90 on both media.

It is also noticed that the mean Cq values of PRM2 did not show any statistically significant difference between the examined temperatures at any time interval on both media.

These results agree with **(14)** who stated that the Ct values of the studied mRNA markers did not show statistically significant differences between samples stored at different temperatures and concluded that humidity and light affect mRNA degradation more than temperature.

Regarding the comparison between cotton and glass, the mean Cq values of PRM2 did not show any statistically significant difference at the different time intervals and temperatures studied. Additionally, the mean Cq values of PRM1 were statistically significantly higher ($P < 0.05$) on cotton media than on glass media at room temperature (at day 0) and 4°C (at both days 0 and 45). These findings indicate that PRM2 is a reliable mRNA marker that can be used to estimate the time since deposition of semen on different media across temperatures as its degradation pattern was not affected by media differences.

Unfortunately, we did not find any previous study comparing the degradation patterns of mRNA markers of body fluids on porous *versus* non-porous surfaces.

Conclusion

These findings reveal that PRM2 is a better mRNA marker compared to PRM1 that can be used to estimate the time since deposition of semen stains at different temperatures and on different media, as the mean Cq values of PRM2 at each time interval did not show statistically significant difference between temperatures while PRM1 showed statistically significant differences in its mean Cq values between temperatures at day 45 on the two examined media. In addition, the mean Cq values of PRM2 at each time interval did not show statistically significant difference between different media while the mean Cq values of PRM1 were significantly higher on cotton media at day 0 at room temperature and 4°C, and at day 45 at 4°C.

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