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Research Paper

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 messenger Ribonucleic Acid (mRNA) can be used to identify the type of body fluid, its degradation can also indicate the time interval since it was deposited. This study was conducted to evaluate the effect of temperature on the estimation of the age 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 protamine 1 (PRM1) and protamine 2 (PRM2) were quantitatively assessed along with a reference gene, beta-actin, using a 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 in 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 in both media.

Conclusion: These results indicate that PRM2 can act as a reliable mRNA marker to estimate the time of deposition of semen stain at different temperatures on two different media.

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1. Introduction

Some Ribonucleic Acid (RNA) forms, such as messenger (m)RNA can disclose the activities of various genes and the identity of the corresponding 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 the deposition of information is critical for criminal investigations. Knowing when body fluid was deposited at a crime scene can help investigators determine when the crime occurred. Additionally, the deposition of samples which do not match the time of the crime 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 a major problem for investigators. Degradation passively influences the nucleic acid containing all the information that can help solve 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 when the crime occurred [4].

2. Materials and Methods

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 the 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 the Faculty of Medicine of Cairo University.

Study population

Participants

A total of 10 semen samples with normal quantity and quality were collected from healthy men aged 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. In the first portion, 50 µL of semen was spotted onto nine pieces of sterilized white cotton cloth (5×10 cm) as an example of the 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. In the second portion, 50 µL of each semen sample was spotted onto nine pieces of sterilized glass slides (5×10 cm) as an example of a 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 ranged from 14°C–25°C.

The mRNA Markers protamine 1 (PRM1) and protamine 2 (PRM2) and the reference gene beta-actin were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at different time intervals of 0, 45, and 90 days.

Ribonucleic Acid (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 reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RT-qPCR assays were conducted with ViPrime One Step RT-qPCR 2×SyGreen Mix (HRox, cat. no QR8602-100, Malaysia) using StepOne Real-Time PCR Applied Biosystems detection system operating v.3.1 of the software (StepOne™, USA). The study was conducted using 5 µL of total RNA and 1 µL of gene-specific primer. The

Table 1. The primer sequences for the measured genes were

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′

thermal cycler settings were 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 extending 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 (Table 1).

Expression values of the studied markers

After performing the RT-qPCR, the data were expressed in the 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 SPSS software, v. 26. To compile the data for quantitative variables and frequencies, the mean and standard deviation were employed. When comparing two groups, student's unpaired 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 the correlation between quantitative variables. P values less than 0.05 were considered statistically significant.

3. Results

On the glass media, the comparison between the different markers at each time interval examined showed that the mean Cq values of the messenger ribonucleic acid (mRNA) markers PRM1 and PRM2 and reference gene beta-actin increased significantly with the time of storage reaching the 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 2).

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 3). The Cq value was higher at room temperature at 40°C versus 4°C for PRM1 and beta-actin (Table 4).

On the cotton media, the 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 increased significantly with the time of storage, reaching the highest levels at day 90 (P<0.001). This increase was observed at different storage temperatures (room temperature, 4°C, and 40°C) (Table 5).

Comparing the mean Cq values of each marker at different temperatures on cotton media showed that the only marker presenting a statistically significant difference in its mean Cq values was PRM1 at day 45 (P<0.05) (Table 6). Its mean Cq value was higher at room temperature than at 4°C (Table 7).

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 in cotton was significantly higher than in glass media at day 0, while beta-actin levels were significantly higher in glass media (P<0.05). Otherwise, no significant difference was observed between media in the mean Cq of any marker at any time interval (Table 8).

At 4°C, a statistically significant difference was observed between glass media and cotton media in the mean Cq value of PRM1 on days 0 and 45 (with higher values in cotton media) (P<0.05). Besides, no statistically significant difference was observed between media in the mean Cq of any marker at any time interval (Table 9).

Table 2. Comparison between different markers at each time interval on Glass media

		Mean±SD			P
		day 0	day 45	day 90	
Room Temperature	PRM1	24.45±3.58	36.69±2.97	40±4.65	<0.001
	PRM2	20.33±3.66	29.49±3.45	34.25±4.85	<0.001
	Beta actin	24.63±2.52	35.74±3.31	38.17±4.26	<0.001
	P	0.010	<0.001	0.028	
4°C	PRM1	23.04±4.14	32.64±2.41	37.27±3.76	<0.001
	PRM2	19.39±3.26	25.64±5.91	31.77±4.73	<0.001
	Beta actin	23.33±3.56	31.79±4.23	35.44±3.18	<0.001
	P	0.042	0.003	0.014	
40°C	PRM1	23.97±3.75	37.69±1.93	39.47±3.28	<0.001
	PRM2	20.20±3.48	24.79±5.74	34.94±2.93	<0.001
	Beta actin	22.25±3.18	35.44±3.22	36.92±3.37	<0.001
	P	0.070	<0.001	0.014	

P value is statistically significant (P≤ 0.05)

Comparing the mean Cq value of all markers in both media (glass and cotton) at each time interval at 40°C also indicated that no statistically significant difference was observed between media in the mean Cq value of any marker at any time interval (Table 10).

4. Discussion

Biological evidence collected from crime scenes can be affected by various environmental conditions, while

Table 3. Comparison between different temperatures for each marker at day 0, day 45 & day 90 on Glass media

Days		Mean±SD			P
		Room Tm	4°C	40°C	
0	PRM1	24.45±3.58	23.04±4.14	23.97±3.75	0.708
	PRM2	20.33±3.66	19.39±3.26	20.20±3.48	0.808
	Beta actin	24.63±2.52	23.33±3.56	22.25±3.18	0.249
Day 45	PRM1	36.69±2.97	32.64±2.41	37.69±1.93	<0.001
	PRM2	29.49±3.45	25.64±5.91	24.79±5.74	0.114
	Beta actin	35.74±3.31	31.79±4.23	35.44±3.22	0.038
Day 90	PRM1	40±4.65	37.27±3.76	39.47±3.28	0.276
	PRM2	34.25±4.85	31.77±4.73	34.94±2.93	0.234
	Beta actin	38.17±4.26	35.44±3.18	36.92±3.37	0.260

P is statistically significant (P≤ 0.05)

SD: Standard deviation

Table 4. Pairwise comparison between Room Temperature, 4°C & 40°C for each marker at different time intervals on Glass media

		Room Tm vs 4°C	Room Tm vs 40°C	4°C vs 40°C
Day 45	PRM1	0.003	1.000	< 0.001
	Beta actin	0.044	1.000	0.097

P is statistically significant (P≤ 0.05)

the surroundings are expected to change the kinetics of RNA degradation [7].

This study was conducted to evaluate the effect of temperature on estimating the time of deposition of semen on a porous surface (cotton) versus a 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 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 three temperatures and in both media. These are consistent 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 during the 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 at 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, such as GAPDH, ACTB, and

Table 5. Comparison between different markers at each time interval on Cotton media

		Mean±SD			P
		Day 0	Day 45	Day 90	
Room temperature	PRM1	28.08± 3.43	38.83± 2.48	39.62± 3.68	<0.001
	PRM2	18.98± 3.11	30.21± 6.55	34.86± 4.36	<0.001
	Beta actin	21.62± 3.13	36.34± 3.81	37.69± 4.74	<0.001
	P	<0.001	0.001	0.060	
4°C	PRM1	27.03± 3.15	35.91± 2.76	37.45± 1.99	<0.001
	PRM2	18.16± 3.15	26.37± 4.74	33.70± 2.45	<0.001
	Beta actin	21.61± 2.70	34.69± 4.46	36.61± 3.28	<0.001
	P	<0.001	<0.001	0.009	
40°C	PRM1	24.21± 4.02	37.12± 2.16	39.63± 4.28	<0.001
	PRM2	19.79± 5.10	28.61± 4.20	34.15± 3.64	<0.001
	Beta actin	23.11± 5.02	35.18± 3.57	35.88± 3.50	<0.001
	P	0.113	<0.001	0.011	

P is statistically significant (P≤ 0.05)

Table 6. Comparison between different temperatures for each marker at day 0, day 45 & day 90 on Cotton media

Days		Mean±SD			P
		Room Tm	4°C	40°C	
0	PRM1	28.08±3.43	27.03±3.15	24.21±4.02	0.057
	PRM2	18.98±3.11	18.16±3.15	19.79±5.10	0.650
	Beta actin	21.62±3.13	21.61±2.70	23.11±5.02	0.595
45	PRM1	38.83±2.48	35.91±2.76	37.12±2.16	0.045
	PRM2	30.21±6.55	26.37±4.74	28.61±4.20	0.278
	Beta actin	36.34±3.81	34.69±4.46	35.18±3.57	0.638
90	PRM1	39.62±3.68	37.45±1.99	39.63±4.28	0.284
	PRM2	34.86±4.36	33.70±2.45	34.15±3.64	0.767
	Beta actin	37.69±4.74	36.61±3.28	35.88±3.50	0.585

P value is statistically significant (P≤ 0.05)

SD: Standard deviation

Table 7. Pairwise comparison between Room Tm , 4°C & 40°C for each marker at different time intervals on Cotton media

			Room Tm vs 4°C	Room Tm vs 40°C	4°C vs 40°C
Cotton	Day 45	PRM1	0.042	0.404	0.855

P is statistically significant (P≤ 0.05)

Table 8. Comparison between mean Cq of markers on both media (Glass versus Cotton) at each time interval at Room Temperature

Days		Mean±SD		P
		Glass	Cotton	
0	PRM1	24.45±3.58	28.08±3.43	0.032
	PRM2	20.33±3.66	18.98±3.11	0.386
	Beta actin	24.63±2.52	21.62±3.13	0.029
45	PRM1	36.69±2.97	38.83±2.48	0.097
	PRM2	29.49±3.45	30.21±6.55	0.762
	Beta actin	35.74±3.31	36.34±3.81	0.711
90	PRM1	40.00±4.65	39.62±3.68	0.842
	PRM2	34.25±4.85	34.86±4.36	0.771
	Beta actin	38.17±4.26	37.69±4.74	0.815

P is statistically significant (P≤ 0.05)

Table 9. Comparison between mean Cq of markers on both media (Glass versus Cotton) at each time interval at 4°C

Days		Mean±SD		P
		Glass	Cotton	
0	PRM1	23.04± 4.14	27.03± 3.15	0.026
	PRM2	19.39± 3.26	18.16± 3.15	0.402
	Beta actin	23.33± 3.56	21.61± 2.70	0.239
45	PRM1	32.64± 2.41	35.91± 2.76	0.011
	PRM2	25.64± 5.91	26.37± 4.74	0.764
	Beta actin	31.79± 4.23	34.69± 4.46	0.153
90	PRM1	37.27± 3.76	37.45± 1.99	0.895
	PRM2	31.77± 4.73	33.70± 2.45	0.267
	Beta actin	35.44± 3.18	36.61± 3.28	0.428

P is statistically significant (P≤ 0.05)

B2M can be studied to estimate sample age. This study concluded that a global decrease is observed in mRNA abundance in aging stains.

These results were following [12] who studied the applicability of using RNA degradation as a method for detecting the age of stored blood samples using Cq values of mRNA markers. Their study showed that ΔCq values of dried blood samples are increasing with time and can be used as a method to identify 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 only in the glass media. 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,

Table 10. Comparison between mean Cq of markers on both media (Glass versus Cotton) at each time interval at 40°C

Days		Mean±SD		P
		Glass	Cotton	
day 0	PRM1	23.97± 3.75	24.21± 4.02	0.892
	PRM2	20.20± 3.48	19.79± 5.10	0.836
	Beta actin	22.25± 3.18	23.11± 5.02	0.653
day 45	PRM1	37.69± 1.93	37.12± 2.16	0.541
	PRM2	24.79± 5.74	28.61± 4.20	0.107
	Beta actin	35.44± 3.22	35.18± 3.57	0.866
day 90	PRM1	39.47± 3.28	39.63± 4.28	0.926
	PRM2	34.94± 2.93	34.15± 3.64	0.600
	Beta actin	36.92± 3.37	35.88± 3.50	0.507

P is statistically significant (P≤ 0.05)

20°C, and 37°C) and revealed that the mean Cq values of RNA transcripts raised with increasing temperatures.

Also, Hermann [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 following [9] who concluded that Ct values of studied markers were higher in aged samples at room temperature compared to 35°C and this finding was explained by an effect of humidity and light at room temperature on RNA degradation.

According to the study, no statistically significant difference was observed in the mean Cq values of PRM1 and beta-actin between the examined temperatures on days 0 and 90 in 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 are consistent 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 ($P<0.05$) in cotton media than in 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 at temperatures because its degradation pattern was not affected by media differences.

Unfortunately, we found no previous study comparing the degradation patterns of mRNA markers of body fluids on porous versus non-porous surfaces.

5. Conclusion

These findings show that PRM2 is a better mRNA marker compared to PRM1 that can be used to estimate the time of deposition of semen stains at different temperatures and on different media because the mean Cq values of PRM2 at each time interval did not show a 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 a 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.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed about the purpose of the research and its implementation stages; they were also assured about the confidentiality of their information; Moreover, They were allowed to leave the study whenever they wish, and if desired, the results of the research would be available to them

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Authors' contributions

Responsible for establishing the experimental design of the research, starting from the idea, doing the experimental work, and assisting in the paper writing: Basma Kamal Ibrahim; Assisting in the interpretation of the results and critical revision of the paper: Abla Abdelmeguid Attia; Responsible for the biochemical and molecular workup of the research: Mohamed Ahmed Abd El Salam; Assisting in establishing the experimental design of the research and the final revision: Laila Ahmed Rashed; Responsible for the interpretation of results and the paper writing: Heba Abdo Abdel Razik; all authors read and approved the final manuscript.

Conflict of interest

The authors declared no conflict of interest.

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