Applications of RNA based methods in forensic medicine

Doctoral (PhD) thesis

Viktor Soma Poór

Supervisor: Dr. Katalin Sipos
Head of Doctoral Program: Dr. Attila Miseta
Head of Doctoral School: Dr. Gábor L. Kovács

University of Pécs
Medical School
Pécs

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Introduction

Classic methods for estimation of post-mortem interval (PMI)

The post-mortem interval is the time period between someone's death and the finding of the cadaver. Estimation of PMI is one of the most researched field in forensic medicine.

All the estimations based on the classic signs of death (livores mortuales, rigor mortis, algor mortis) suffer from a high degree of uncertainty, also can be influenced by several internal and external factors.

Forensic entomology might provide an estimation based on the colonisation of the cadaver by insects in the case of elongated PMI. Specialised expertise is necessary for this kind of examination. Also several factors can influence the precision of the estimation.

PMI in the context of RNA degradation

DNA is significantly more stable than RNA. Structural differences and the omnipresence of RNase enzymes can explain this deviation.

Setzer et al. studied the detectability of RNA in blood, saliva and semen stains and in vaginal swabs. They proved, if the conditions are favourable (room temperature, dry air, hidden from direct light) it is possible to isolate RNA from some of the samples after 547 days. However samples subjected to harsh environmental conditions showed drastic degradation.

Sampaio-Silva and colleagues studied the effect of PMI on RNA degradation in mouse skeletal muscle model. They investigated the change of mRNA levels of general housekeeping genes (*Actb, Gapdh*) and genes specific to skeletal muscle with quantitative real-time PCR. Normalised to the most stable *Rps29* mRNA (coding a ribosomal protein) levels, the Ct of the other genes proportionally increased with PMI. They created a mathematical model from the data, what was used to predict PMI with high precision up to nine hours. The practical application of such a short interval is limited.

Several other research projects were recently established with a goal of developing RNA based applications for forensic purposes. Most of these studies focused on determination of origin of biological fluids and stains. In multicellular organisms the genomic DNA content is the same in every somatic cells (excluding the mutations), but the transcriptome of tissues is markedly different. The identification of mRNA profile with PCR can help to identify the stain in question.

Estimation of PMI based on the examination of teeth

There are seldom any data on post-mortem changes of the teeth in the scientific literature. Microscopic examination of cadaveric teeth revealed that the number of living odontoblasts decreased to zero after five days.

Short term RNA stability was measured in healthy teeth, extracted from patients. RNA was kept at room temperature, 4 °C, -20°C and -80 °C for a time ranging from 0 to 24 hours. The isolated RNA from the tooth pulp showed no significant degradation with gel electrophoresis.

Young et al. estimated PMI from extracted porcine teeth. They compared the ratio of 70 and 300 base pair PCR products of ß-actin mRNA in relation to PMI. Their results showed that the degradation of the longer fragments were significantly faster than the 70 base pair product. The change of the ratio of the two fragments with time was not linear. The precision of the PMI estimation was improved by colorimetric measurement of the pulp tissue.

Summary

- One of the most important tasks of forensic medicine is the determination of post-mortem interval (PMI).
- Several methods were developed, which can:
    - only estimate PMI.
    - be used only in a given timeframe (hours – days: classic signs of death; days – months: entomology; months – years: anthropology).
- Several studies aimed to quantify RNA degradation and estimation of PMI from this data.
- The use of teeth in forensic medicine is widespread.
- Teeth are impervious to environmental factors, what can limit their influence on the rate of RNA degradation.

Aims

I have studied the possibility of RNA based applications in forensic medicine. I aimed to develop a method of PMI estimation based on RNA degradation. I investigated the following topics:

- Develop methods which are able to measure RNA degradation in forensic samples.
- Decide which tissue is suitable for PMI estimation.
- Develop a sampling protocol.
- Determine the interval range and precision of RNA degradation based PMI estimation.

Materials and methods

Collection and storage of blood samples

After an informed consent, venous blood was drawn from healthy volunteers into native BD collecting tubes. Fifty microliter aliquots were immediately pipetted on cellulose filter paper. The droplets were left to dry completely then stored under dry and dark condition at room temperature.

Extraction, storage of teeth. Extraction of pulp tissue

Intact premolars and molars were offered for scientific purposes after an informed consent. The teeth were removed due to orthodontic reasons. The extracted but still intact teeth were stored at room temperature (20 °C – 25 °C), kept away from light.

After incubation the teeth were cut into half under constant water cooling with a dental burr (15,000 rpm). The extracted pulp tissue was transferred into sterile sample tube and kept at -70 °C until RNA isolation.

RNA isolation and reverse transcription

For RNA isolation I used RNeasy Micro Plus (Qiagen) kit with slight modification from the manufacturer's protocol. The samples were treated with DNase enzyme on column.

The reverse transcription (cDNA synthesis) was performed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations.

Polymerase chain reaction (PCR)

For DNA amplification I used iProof High-Fidelity DNA Polymerase (Bio-Rad) kit, which contains phusion DNA polymerase derived from *Pyrococcus furiosus.* The reaction setup followed the manufacturer's recommendations.

Determination of RNA degradation

The determination of RNA integrity was performed by an Agilent 2100 Bioanalyzer instrument, using RNA 6000 Pico total Eukaryotic microfluidic chip (Agilent). The data analysis was performed with 2100 Expert software (Agilent).

Results

Age determination of bloodstains

Equal amounts of fresh, native blood was pipetted onto untreated filter paper. The samples were stored at room temperature.

Fragments (200 base pair) of human ß-actin mRNA and 28S rRNA were amplified by quantitative real-time PCR. I set up a hypothesis as due to its secondary structure the ribosomal RNA will degrade less than the messenger RNA. The change of the ratio between the two PCR products might be able to tell the PMI.

First RNA was isolated immediately after the sample dried. Following the cDNA synthesis, Ct values of ß-actin and 28S RNA were determined.

Ct ß-actin	Ct 28S rRNA	ΔCt (Ct ß-actin - Ct 28S rRNA)
32.25	28.11	4.14
33.79	28.55	5.24
32.84	29.8	3.04
33.06	27.79	5.27
33.65	31.95	1.7
35.91	31.6	4.31
25.78	19.74	6.04
26.55	20.59	5.96
28.41	25.8	2.61

Table 1. *Results from bloodstains with no incubation.*

Table 1. shows that the differences between the samples are pronounced, even without any incubation. Apart from the high variability of the individual Ct values, the ratio of the two housekeeping genes vary between 1.7 and 6.04.

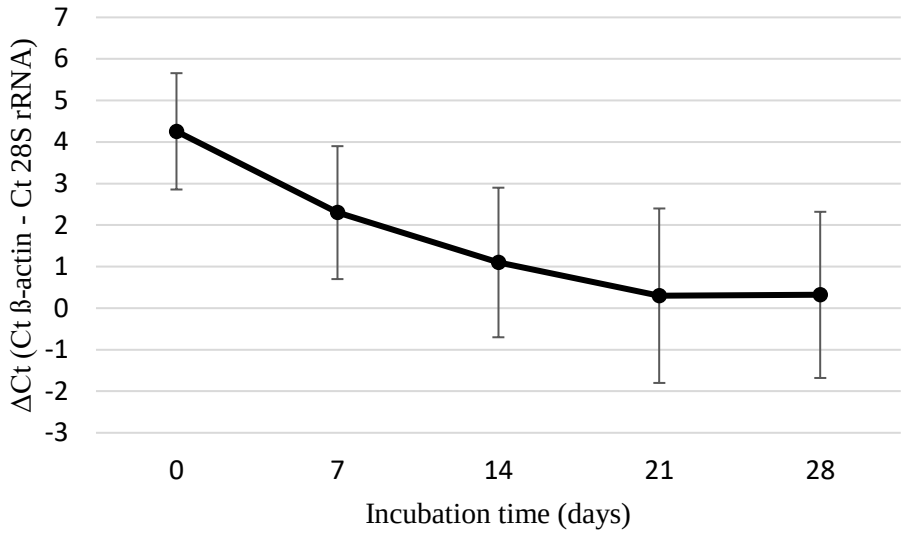

Figure 1. *Change of ratio of the Ct values of ß-actin and 28S rRNA after incubation at room temperature. Datapoints show the means of Ct ratios, error bars show the standard deviation (SD).*

The bloodstains were incubated for one to four weeks. After reverse transcription Ct values of ß-actin and 28S rRNA were determined. The difference of the two Ct values of each samples are shown at Figure 1. We can see the trend of decreasing difference, but the difference between time points was not significant (ANOVA). The conclusion is that this method alone cannot be used to estimate the time since deposition of the blood droplet.

Determination of RNA integrity with Agilent 2100 Bioanalyzer

For the determination of RNA integrity, the Agilent 2100 Bioanalyzer was used. The instrument contains the electrodes and fluorescent detector. The separation of RNA molecules according to size is done on disposable chips.

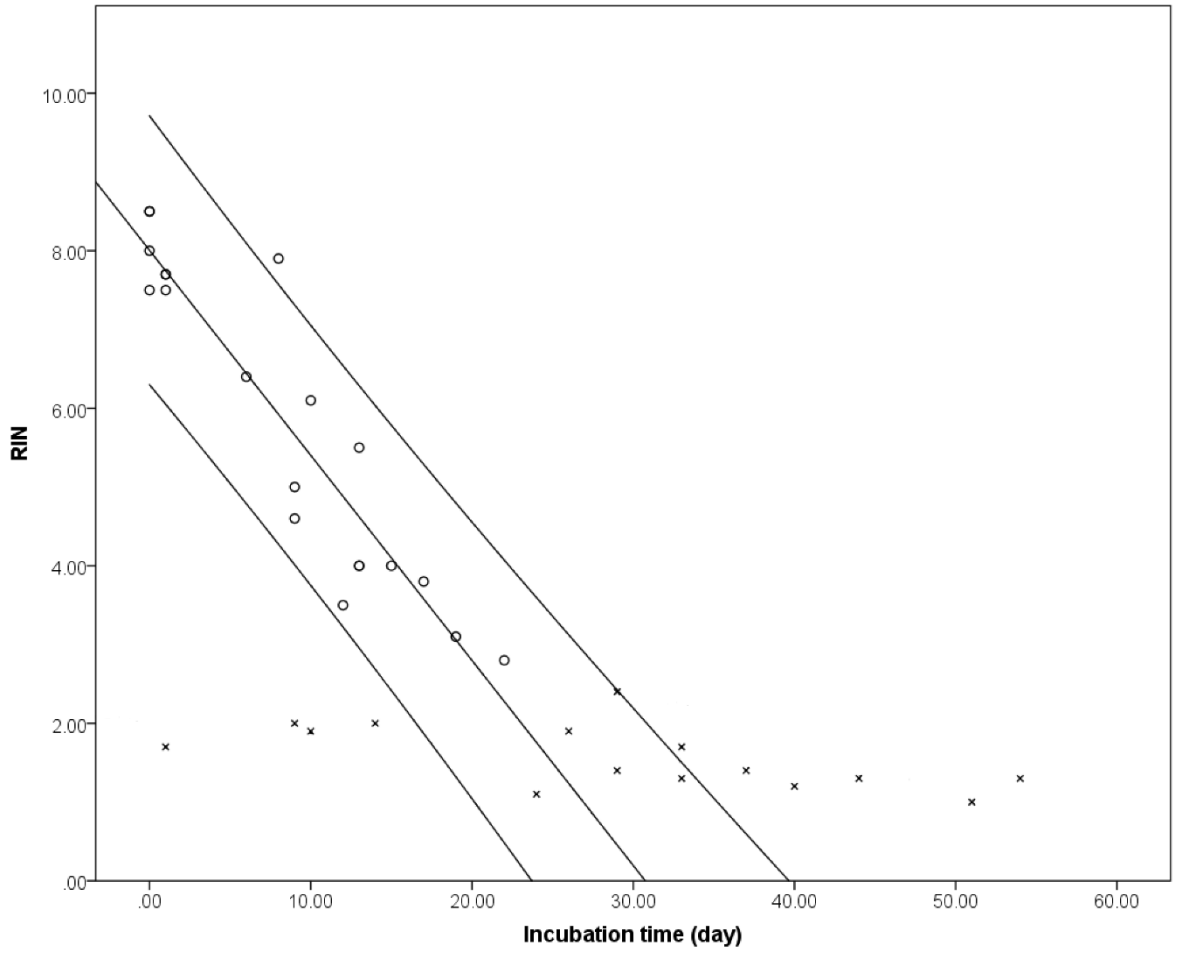

Figure 2. *Change of RNA integrity in relation to incubation time. The best fit line and the 95% confidence interval is shown. The data points below the cut-off value are marked with "x".*

The figure above shows that the decline of RIN is linear with incubation time. All the samples which were incubated for 25 days or more showed advanced degradation, RIN values did not correlate with incubation time. I determined a cut-off value (RIN < 2.4). The samples below this threshold were excluded from statistical analysis.

The equation of the best fit line is Y=8.005-0.26X, where Y is the incubation time in days, X is the RIN values. The fit of the line is R^2=0.859. The correlation between the two parameters is statistically significant (P <0.001). The upper and lower boundaries of 95% confidence interval can be described by the following equations: Y=7.435-0.313X and Y=8.575-0.208X.

Tooth pulp RT-PCR

It is possible to amplify degraded cDNA with PCR. My hypothesis is that the detection of longer PCR products decline more with time than the detection of short PCR products.

For target I chose 800, 400 and 200 base pair long segments of human ß-actin mRNA and 28S rRNA isolated from teeth pulp. These genes are housekeeping genes, what means that the change of detectability can be explained only with degradation.

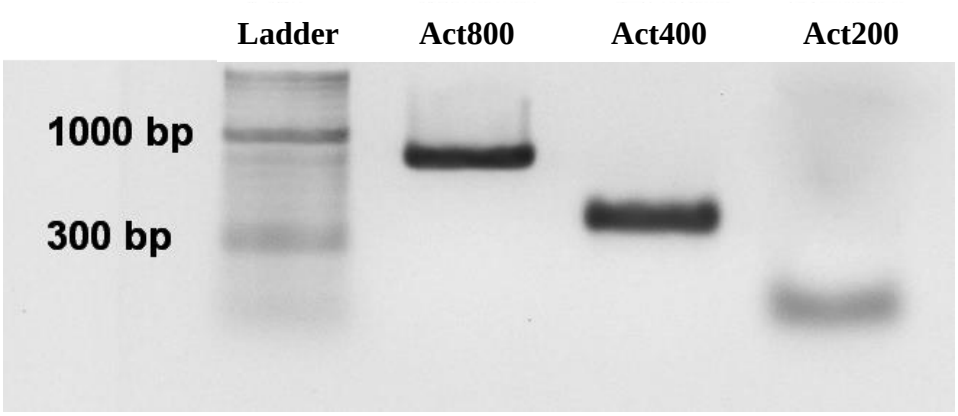

Figure 3. *800, 400 and 200 base pair long amplicons on agarose gel.*

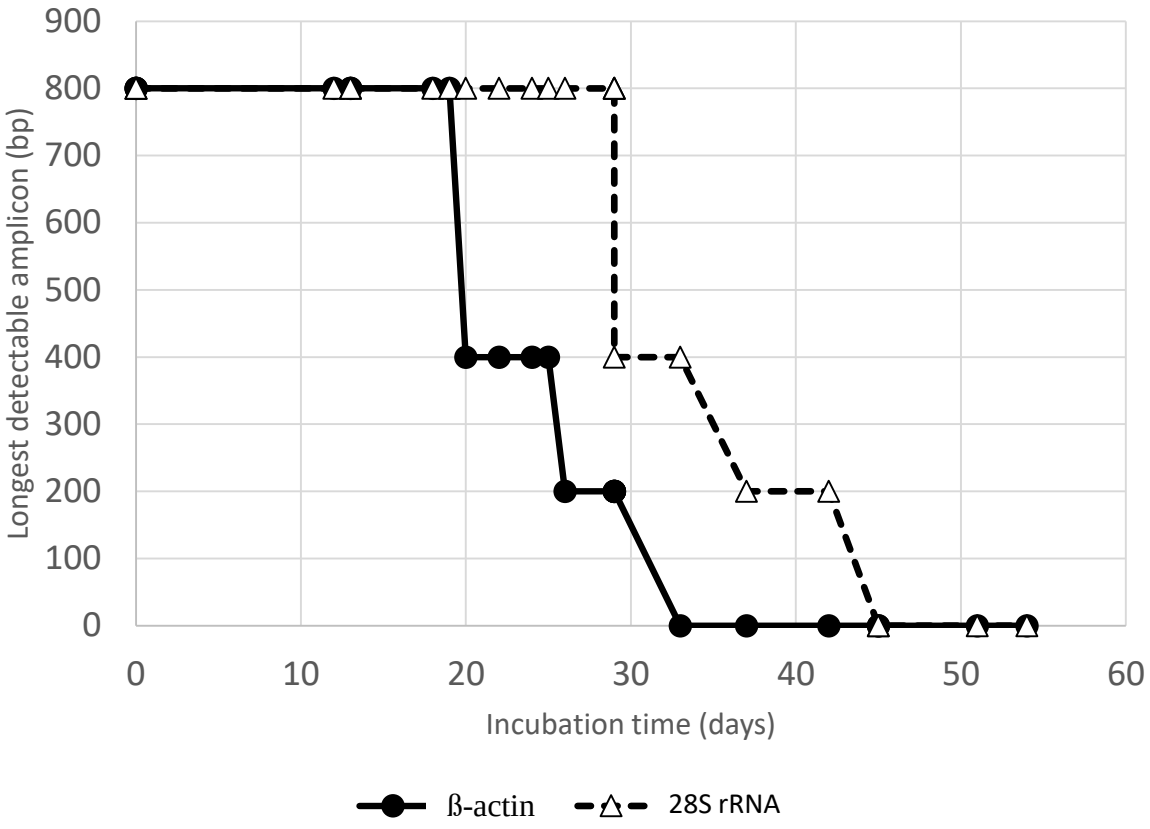

Figure 4. *The detectable PCR amplicons from cDNA.*

Figure 4. summarizes the detectability of different sized PCR products from the pulp of incubated teeth.

Validating the results of tooth pulp

For the validation of the results I asked the contributing dentist to collect and incubate teeth without revealing the date of the extraction. The total number of these teeth were six. The RIN and PCR measurements were performed as described above.

Out of these six teeth in one case the measurement gave no RIN value, in another case the RIN value was 2.1, which is below the cut-off limit. These two samples were excluded from the calculations.

Summarizing the data from RIN and PCR measurements, I performed the calculations to estimate the incubation time and the 95% confidence interval. At those cases where RIN value was available, the PCR results confirmed them. Comparing the calculated incubation time with the real incubation time all four cases were within the boundaries of the 95% confidence interval.

Sample	RIN	Detected amplicon		Estimation of incubation time in days (95% confidence interval)	Incubation time (days)
		ß-actin mRNA	28S rRNA		
1. sample	3.1	800 bp	800 bp	18.8 (14-26)	17
2. sample	7.5	800 bp	800 bp	1.9 (0-5)	5
3. sample	2.1	200 bp	800 bp	26-29	26
4. sample	6.4	800 bp	800 bp	6.2 (3-10)	7
5. sample	3.4	800 bp	800 bp	17.7 (13-25)	15
6. sample	n.d.	n.d.	200 bp	33-42	38

Table 2. *Comparison of the estimated and the real incubation intervals. n.d.: non detected*

Examination of pathological teeth

The question arose whether the various pathological conditions of teeth affect the quality and quantity of tooth pulps.

I examined twenty pathological teeth, which were extracted due to various reasons. From half of these teeth the pulp tissue was not enough for RNA isolation. Out of these ten teeth two suffered from acute periodontitis, three of chronic parodontitis. The caries reached the pulp chamber in five of the teeth.

Table 3. shows that out of the ten remaining teeth only three contained sufficient amount of mRNA to amplify ß-actin. I found no correlation between the indication of extraction and the RIN/PCR results.

Indication of extraction	RIN value	200 bp amplicon	400 bp amplicon	800 bp amplicon
acute periodontitis	1,3	Positive	Positive	Negative
prosthetic	1	Negative	n.a.	n.a.
periostitis, cysta radicularis,	1	Negative	n.a.	n.a.
acute periodontitis	n.d.	Negative	n.a.	n.a.
acute periodontitis	2,4	Positive	Positive	Negative
prosthetic	3,2	Positive	Positive	Negative
Prosthetic	n.d.	Negative	n.a.	n.a.
acute parodontitis	n.d.	Negative	n.a.	n.a.
acute periodontitis	n.d.	Negative	n.a.	n.a.
prosthetic	n.d.	Negative	n.a.	n.a.

Table 3. *Summary of results of pathological teeth. The longest detectable ß-actin mRNA fragments n.d.: non detectable n.a.: non available, the measurement was not performed.*

Summary

- There were no significant correlation between the PCR results and PMI in bloodstains.
- The decrease of RNA integrity showed close correlation with incubation time until the 25^{th} day.
- By the detection of different length PCR amplicons it is possible to estimate incubation time until the 42^{th} day.
- Performed the method's validation on six teeth samples.
- Pathological teeth showed increased degradation.

Discussion

Estimation of bloodstains' age

It is obvious from our experimental results, that with the utilised methods the age prediction of bloodstains showed large variance.

RNA isolation from blood usually yield low concentration, even under ideal circumstances. RNA concentration was under 3 ng/µl from fresh samples. At low template concentration the stochasticity of PCR dramatically increases. In quantitative real-time PCR the variability of Ct values increases over 30. For this reason, many of the evaluation software exclude those results from the calculations. Most of the Ct values of the incubated bloodstains exceeded that threshold.

Blood seems to be a simple tissue. However, the number and the composition of white blood cells change dynamically, answering infections or inflammatory conditions. This can explain interpersonal and intrapersonal differences.

Despite the normalised conditions slight changes of environmental factors, such as temperature, humidity, might have contributed to the high variance of results.

Because of the above mentioned reasons, this method cannot be used to estimate reliably the age of bloodstain.

Estimation of PMI by measuring RNA degradation of tooth pulp

Measuring RNA integrity

Dentin and enamel are among the most resilient tissues of the human body. These layers provide a high degree of isolation from environmental factors and mechanical impact. This makes tooth pulp tissue a promising candidate for PMI estimation.

The microfluidics based Agilent Bioanalyzer is one of the fastest way to measure RNA degradation. The quantity and to some extent, the quality of RNA can be determined by classic methods, for example UV spectrum recording, RNA agarose gel electrophoresis, but

the results are difficult to quantify. The microfluidic platforms showed high correlations with quantitative real-time PCR results.

In our samples RNA integrity number showed a linear correlation with the incubation time of teeth. The following equation can give estimation of incubation time: Y=8.005-0.26X, where Y is the time of incubation in days, X is the RIN value. The fit of the line is R^2=0.859. Samples of more than 25 days incubation had too low RIN values for estimation.

PCR based investigations

Up to 25 days the microfluidic method can give precise estimation of incubation, what can be extended to 42 days with a PCR based method.

Detectability of ribosomal and messenger RNA amplicons starts to differ after 19 days, what can improve the accuracy of estimation. The distinction can be explained by two mechanisms. Ribosomal RNA species maintain complex secondary structures, what decreases the efficiency of RNase enzymes. On the other hand, the sheer number of ribosomal RNAs can help with detection.

The parallel applications of microfluidic and PCR based methods is highly recommended. It can enhance the accuracy and expand the timeframe of estimation. Also, the PCR results can verify the microfluidic results. An estimation can be considered technically valid only if both methods gave the same result.

Limitations of tooth pulp examinations

In this pilot study, extracted teeth were used as experimental model. Even that the tooth pulp is highly isolated, the conditions of a corpse are not completely identical to our model experiment. These results should not be extrapolated to cadaveric teeth without additional verification.

It is known, that temperature has high impact on degradation of biomolecules. In these model experiment I could only use a limited amount of teeth, therefore grouping them into subgroups of different temperatures, would render the statistical analysis futile, due to the low

sample sizes. With more teeth it is possible and desirable to study the effects of temperature on RNA degradation.

The poor dental hygiene of the population might hinder the practical application of the aforementioned methods. The DMFT index, what shows the number of decayed, missing, filled teeth is high, 16.04 in the Hungarian population. 38.7% of the residents over 75 years has lost all their natural teeth. These numbers are in concordance with the world average.

Experiments with teeth affected by dental diseases suggest that pathological conditions influence the state of tooth pulp. There was no correlation between the rate of accelerated degradation and the type of the pathological condition, except progressed caries, where the damage reached to pulp chamber.

The methods described above seem promising in estimation of post-mortem interval by measuring RNA degradation in tooth pulp. No method is able to predict PMI with 100 percent confidence, but results of multiple techniques can increase the precision and accuracy of PMI estimation.

Summary of new results

- The tested method could not estimate reliably the age of bloodstains. Reasons could be:
    - high variability due to low RNA concentrations
    - variability between samples
    - incubation conditions
- The incubation interval was estimated with high precision by measuring RNA integrity of tooth pulp.
- The PCR based method could:
    - extend the range of estimation to 42 days.
    - verify the data of microfluidic measurement.
- Performed the internal validation of the method. The estimations for all the six tested teeth were within the boundaries of 95% confidence interval.
- Teeth affected by pathological conditions showed accelerated degradation.

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List of scientific publications and conference presentations

Cumulative impact factor: 21,379
Cumulative citations: 34
Independent citations: 29

Thesis is based on the following scientific publication

V.S. Poór, D. Lukács, T. Nagy, E. Rácz, K. Sipos: The rate of RNA degradation in human dental pulp reveals post-mortem interval
International Journal of Legal Medicine. 130(3):615-619. 2016 IF: 2,714

Additional scientific publications

E. Rácz, F. Könczöl, D. Tóth, Z. Patonai, Z. Porpáczy, Z. Kozma, **V.S. Poór**, K. Sipos.: PCR-based identification of drowning: four case reports
International Journal of Legal Medicine. 130(**5**):1303-1307. 2016 IF: 2,714

E. Rácz, F. Könczöl, H. Mészáros, Z. Kozma, M. Mayer, Z. Porpaczy, **V.S. Poór**, K. Sipos: Drowning-related fatalities during a 5-year period (2008-2012) in South-West Hungary - A retrospective study
Journal of Forensic and Legal Medicine 31():7-11 2015 IF: 0,76

A. Miseta, J. Nagy, T. Nagy, **V.S. Poór**, Z. Fekete, K. Sipos: Hepcidin and its potential clinical utility
Cell Biology International 39(**11**):1191-1202 2015 IF: 1,933

T. Nagy, D. Frank, E. Kátai, R.K.K. Yahiro, **V.S.** Poór, G. Montskó, Z. Zrínyi, G.L. Kovács, A. Miseta: Lithium induces ER stress and N-glycan modification in galactose-grown Jurkat cells
PLoS ONE 8(**7**):e70410. 2013 IF: 3,354

E. Pandur, K. Sipos, L. Grama. J. Nagy, **V.S. Poór**, G. Sétáló, A. Miseta, Z. Fekete: Prohepcidin binds to the HAMP promoter and autoregulates its own expression
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J. Nagy, L. Lakner, **V. S. Poór**, E. Pandur, G. Mózsik, A. Miseta, K. Sipos: Serum prohepcidin levels in chronic inflammatory bowel diseases
Journal of Crohn's and Colitis 4(**6**):649-653 2010 IF: 1,729

E. Pandur, J. Nagy, **V. S. Poór**, Á. Sarnyai, A. Huszár, A. Miseta, K. Sipos: Alpha-1 antitrypsin binds preprohepcidin intracellularly and prohepcidin in the serum
FEBS Journal 276(**7**):2012-2021 2009 IF: 3,396

Oral conference presentations

Pediastrum (Chlorophycae) in the diagnosis of drowning - case report
Viktor S. Poór, Katalin Sipos, Zsolt Kozma
2017.09.11-15. 10th International Symposium Advances in Legal Medicine (Düsseldorf)

Nukleinsav izolálás igazságügyi mintákból: nehézségek és lehetőségek
Sipos Katalin, **Poór Viktor Soma**
2016.04.23. Igazságügyi szakértői kötelezően szinten tartó továbbképző tanfolyam (Pécs)

Új generációs szekvenálás az igazságügyben
Sipos Katalin, **Poór Viktor Soma**
2016.04.21. Igazságügyi szakértői kötelezően szinten tartó továbbképző tanfolyam (Pécs)

In vino cocto veritas
Poór Viktor Soma, Mayer Mátyás
2016.05.26-28. Fiatal Igazságügyi Orvosszakértők Fóruma (Szeged)

Vízbefulladás? - Új diagnosztikai módszerek bemutatása egy eseten keresztül
Poór Viktor Soma, Rácz Evelin, Simon Gábor, Heckmann Veronika, Sipos Katalin, Kozma Zsolt
2016.01.15. Magyar Igazságügyi Orvosok Társasága (MIOT) kazuisztikai tudományos nap (Budapest)

RNS igazságügyi alkalmazásai
Poór Viktor Soma, Lukács Dénes, Sipos Katalin
2015.09.04 Magyar Igazságügyi Orvosok Társasága (MIOT) XV. kongresszusa (Debrecen)

Posztmortem intervallum becslése RNS degradáció alapján fogbélből
Poór Viktor Soma, Lukács Dénes, Sipos Katalin
2014.10.03. Ifjúsági MIOT gyűlés (Hosszúhetény)

Bort iszik és vizet aspirál
Poór Viktor Soma, Sipos Katalin
2012.10.16. PTE Orvostudományi Szakosztály ülése (Pécs)