Original Research Article

RNA expression pattern for post mortem hair samples- Forensic Implication

Abstract

Aims: The aim of our study is to investigate RNA patterns of a postmortem hair sample. We intend therefore to study the quantitative analysis of Ribonucleic acids quality and quantity.

Study design: This is an experimental and descriptive study which used convenient sampling technique in sample selection.

Methodology: The respondents of this study comprised of living subjects in Rivers State, Nigeria selected through convenient sampling technique.Hair Samples from participants were collected after informed consent using hair tweezers and labelled Ziploc bags. RNA was extracted using the TRI-reagent and its quality and quantity assessed using the Nanodrop.Concentration and purity of RNA were assessed by spectrophotometric analysis using NanoDrop 2000c.

Results:RNA quality seems to diminish with time initially but improved at day 7 and 14, with an improved purity (1.94 and 2.03). However, at a later date the purity diminished further owing to possible degradation with death.RNA Quantity diminishes from 0hrs to 7 days, but rises slowly afterwards from day 14 – 4 months.Pearson's shows negative correlation (r= -0.47) of RNA quality with time.A remarkable feature of this study involved the high linear rate of RNA degradation which resulted in the decrease in RNA quantity within 0-7days. This could be as a

result of increased intracellular enzymatic activity, which is dependent on the natural aqueous environment common to all living cells.

Conclusion: In forensic investigations requiring pure RNA for analysis, this research can be used as an inference to revealing that pure RNA can be obtained some days postmortem, and that hair stands as a good object for case diagnosis.

KEY WORDS: *Hair sample, RNA patterns, RNA quality, RNA quantity, Post mortem analysis, Spectrophotometric analysis*

Highlights:

- Determination of time after death has involved various body fluids and tissues with few studies on use of hair sample. This research thus intend to investigate its relevance.
- Forensic studies in Africa are rare and more so there is paucity of data in studies regarding postmortem changes in Nigeria subjects. This has pegged down forensic case diagnosis.
- Studies involving RNA as a relevant tool for estimation of time after death are few and probably none of such studies exist in Nigeria, hence the need for our study.

1. INTRODUCTION

Estimation of the time since death (TSD) has assisted forensic experts in death investigation and has reduced the investigation time-line and also aided in narrowing down the number of suspects in homicide cases. Numerous researchers have examined various methods for estimating time

since death (TSD) in the past. Although their findings are good and relevant, the authors also affirmed that there are extremely variable and unseen factors, often beyond the control of the investigator in real-time situations, such that the investigator may not comprehend with, and if he will, could eventually lead to a dead-end or much protracted time[1-2]. Sequel to these reports, the researchers advocated for the use of multiple approaches, both qualitative and quantitative, to ensure the reliability and the accuracy of their findings and time being estimated. Some authors have investigated concentration of ions in body fluids, inner eye fluid and other tissue samples including the skin to determine time after death [3-10].Ribonucleic acid analysis for its quality and quantity is a developing technique that has brought solution in estimating post mortem interval. RNA application in forensic science has been widely studied, although the investigations were carried out for various specimen.

RNA detection techniques include the following; agarose gel electrophoresis, northern blot and polymerase chain reaction. Agarose gel electrophoresis is a technique which uses a gel; agarose or sodium dodecyl sulphate [SDS] to separate molecules of a sample subjected to an electric field. The separated molecules form bands which can be recognized by staining and comparing with standard sample bands [11]. Northern blot is non-specific as it cannot discriminate between precursor and mature messenger mRNA thus, is unreliable. Polymerase Chain Reaction is an in vitro DNA amplification procedure in which millions of copies of a particular sequence of DNA can be produced within hours [12-13]. It is one of the evolving quantitative technique used in estimating time since death. Hence Quantitative polymerase chain reaction (qPCR) is of major relevance for nucleic acid detection because it has high specificity and sensitivity.

Degradation of RNA has led to false negative results which has made crime scene detection of time since death an upheaval task [14]. However RNA integrity can be preservedwhen the

activity of Ribonucleases (RNase) enzyme during sample storage is reduced. Thus in thorough preservation, the RNA quality could suggests very relevant information pertaining to the biological data under examiantion. Lindenbergh et al., 2012 [15] in their study used 28 years stored blood and semen stain samples which indicates the long term viability of the RNA. Hence tissue samples if well preserved can offer lots of information especially if the RNA were to be extracted for forensic investigation. Our study therefore aims to develop data for the determination of post mortem interval using hair samples.

Hairs are fine, smooth and slender fibrous outgrowths projecting from the skin that possess different microscopic features such as shape, color, and root appearance[16]. Each hair consists of a shaft and the root. The hair shaft consists of 3 layers: the outermost cuticle, the inner cortex and the medulla. The medulla consists of vacuolated cells filled with air and although they have been reported to occur in larger hairs and as well in medium-sized hairs [16-17].DNA is present in all the body cells, while RNA being the intermediate part of the expression of hereditary information, varies from one body organ to the other[18-19].Therefore the determination of the time since death would entail the measurement of the elevated presence of a particular RNA [20].Juusola et al., 2002[21] proved in their early research that an enormous amount of mRNA can be extracted from biological elements. It has been shown by these authors that co-extraction of DNA and RNA from tissue samples is possible, thus resulting in the availability of sufficient amount for analysis. According to Loureiro et al., 2019 [22], these extractions would require various protocols. For instance, the technique for RNA extraction from bone tissues was suggested by Kuliwaba et al.,2005 [23]and similarly Bradley et al., 2005 [24]reported successful RNA extraction from hair root and documented a successful method for the detection of mRNA after they were preserved for ten days at room temperature after the hairs were plucked.

According to Evans, 2007 [25]in his work on The Casebook of Forensic Detection, hair is considered as a trace evidence at crime scene and as well firm evidence. This is so because the hair of every individual may have many different characteristics, and there may be similarities as well andthis knowledgetherefore could be very useful in supporting other substantial evidence made available at crime scenes. In fact there is hardly any successful forensic investigation without the examination and proper analysis of as many trace elements and even whole body tissues present at the crime scene. Hair analysis has thus provided useful information in estimating post mortem interval as documented by some authors [17 & 26].

2. MATERIAL AND METHODS

A perspective experimental study design was applied in this research study. The respondents of this study comprised of living subjects in Rivers State, Nigeria selected through convenient sampling technique. Prior to obtaining primary data from participants, a participants' consent form was administered and informed consent obtained. Hair Samples from participants were collected using hair tweezers and labelled Ziploc bags**.** RNA was extracted using the TRI-reagent and its quality and quantity assessed using the Nanodrop. The instruments were approved and validated by the Regional Centre for Biotechnology and Bio-resources Research, University of Port Harcourt. The validators examined the instrument alongside the objectives of the study. Test-Retest- Reliability Technique was used in ascertaining instruments reliability on two separate experiments at time T1 and T2. Results of both experiments were correlated. Coefficient of stability was used in ascertaining an excellent reliability of 0.9.

Experimental Procedures

Experimental procedures were carried out using the protocol developed by the Regional Centre for Biotechnology and Bioresources Research, University of Port Harcourt.

- Total RNA was extracted using the method described by Wen et al., (2014)[27] with slight modifications.
- Fifty milligrams (50 mg) of the hair sample was weighed and homogenized with 1 ml Trizol solvent (Invitrogen, USA) and 0.2 ml chloroform. Homogenization was achieved by crushing the hair sample in a sterile ceramic after refrigeration at -20 ^{o} C for 30 minutes.
- The homogenised mixture was thereafter centrifuged at 12,000 r/min for 15 min. Supernatant was decanted and mixed with 0.5 ml 2-propanol, then placed at −80 °C for 30 min to be precipitated.
- The mixture was then centrifuged at 12,000 r/min for 10 min, the supernatant was discarded, and the precipitate was washed with 75% ethanol (3:1 DEPC-H2O).
- The samples were centrifuged and the supernatant was discarded, and then finally, total RNA was dissolved in 60 μL nuclease free water.
- Concentration and purity of RNA were assessed by spectrophotometric analysis using NanoDrop2000c (Erlangen, Germany)

Data was analyzed using the Microsoft Excel data analysis tool 19th Edition. Relationship between RNA Quantity and time interval was expressed using Pearson Correlation.

3. RESULTS& DISCUSSION

According to Mann et al., (1990) [27] post mortem decomposition is affected by environmental factors such as temperature, exposure to rainfall, humidity, composition of the surface on which the body is laid or buried, depth of burial, and the presence of scavengers. In support of this statement, this research was carried out under normal conditions with no exposure to direct sunlight, rainfall, humidity or specific temperatures. This present study allowed for natural decomposition processes without major external influences in order to determine time after death. Hence our study can be relevant in estimating time after death from corpses recovered from enclosed homes and places, or yet deaths occurring without much external influences.

Our study used control samples (sample 2 loading) to regulate results and possible errors and observed that RNA concentration decreased linearly between 0-7 days after death however, at day 14 RNA concentration increased linearly up to 4 months (Table 1).It can be extrapolated that RNA quality seems to diminish with time initially but the improved at day 7 and 14, with an improved purity (1.94 and 2.03). However, at a later date the purity diminished further owing to possible degradation with death.A260nm represents the absorbance of the nucleic acids at wave length of 260 nm and A280nm represents the absorbance of proteins at a wavelength of 280nm. It is deduced from the table that RNA Quantity diminishes from 0hrs to 7 days, but rises slowly afterwards from day 14 – 4 months.

Table 1: Spectrophotometric Analysis of RNA quantity and quality of hair samples

Time (hrs.)	RNA Quantity (ng/µl)	RNA Quality (Purity)

	1st loading	2nd loading	A260(nm)	A280(nm)	260/280
0 hrs.	830.20	793.2	19.83	11.57	1.71
24 hrs.	553.00	564.20	14.11	8.53	1.65
168 (7 days)	377.80	379.30	9.43	4.85	1.94
336 (14 days)	436.70	436.10	10.9	5.37	2.03
2880 (4 months)	563.80	553.20	13.83	8.32	1.66

RNA-Ribonucleic acid, A- Absorbance

Wang et al., 2019 [20] in their study stated that time of death can be estimated by the measurement of the elevated presence of RNA. Another notable feature of this study similar to that observed by Hampson et al., 2011[26] involved the high linear rate of RNA degradation which resulted in the decrease in RNA quantity within 0-7days. This could be as a result of increased intracellular enzymatic activity, which is dependent on the natural aqueous environment common to all living cells. However the degradation rate reduced thereafter causing an increase in RNA quantity at 14 days and 4 months. Pearson's correlation also shows a positive correlation (r=0.55) (Table 2) of RNA quantity with time. But the coefficient of determination (r^2) shows that only about 30% of the samples can account for the correlation seen in our study.

Table 2: Correlation coefficient test for RNA Quantity with time

Time (hrs)	R	R^2	RS
0hrs	0.55	0.30	+ve
24hrs			
168 (7days)			
336 (14days)			
2880 (4months)			

RS- Relationship

Here Bauer et al., (2003) [28] while examining β-actin amplification products, were able to generate a timeline that extended to 60 months under certain conditions. They found that exposure to direct sunlight for two months had no significant effect on the analysis but that samples stored at 37^{o} C yielded no usable RNA. Our study agreed with their results as RNA extracted at 4 months' showed relative amount of RNA. We were able to obtain RNA despite the protracted time after death.

Our study adopted the Beer- Lambert law that draws a direct correlation between absorbance and concentration. While nucleic acids absorb at many wavelengths, they have a peak absorbance of ultra violet light at 260nm. Thus, the amount of light absorbed in this region can be used to determine the concentration of RNA or DNA in solution. The study revealed a correlation between absorbance level of nucleic acids and concentration value; the higher the

absorbance the more the concentration of RNA. However, the Beer- Lambert equation is only linear for absorbances between 0.1 and 1.0. This translates to concentrations between 10.0 ng/uL and 3700 ng/uL when using the Nanodrop ND- 1000. Nucleic acids absorb ultraviolet light at 260 nm due to the aromatic base moieties within their structure. Purines (thymine, cytosine and uracil) and pyrimidines (adenine and guanine) both have peak absorbances at 260 nm, thus making it the standard for quantitating nucleic acid samples. The 280 nm absorbance is measured because this is typically where proteins and phenolic compounds have a strong absorbance.

This study also adopted a correlation between these concentrations and purity level of nucleic acids in consonance with the Beer- Lamberts law. It was observed that the higher the concentration (RNA quantity), the lower the RNA purity (Fig.1). RNA yielded at day 7 and 14 with the least values of RNA quantity of 377.80ng/µl and 436.70 ng/µl respectively were observed to be very pure (Table 4) (Fig.2). However Pearson's correlation of RNA quality with time shows a weak negative correlation (r=-0.47) (Table 3). This infers that RNA quality diminishes with time following death.

Table 3: Correlation coefficient test for RNA Quality with time

Time (hrs)	**R**	**R^2**	**RS**
0hrs			

24hrs	-0.47	0.22	-ve
168 (7days)			
336 (14days)			
2880 (4months)			

RS- Relationship

Although RNA quality was purer on day 7 and 14 after an initial decline in early days, the purity reduced further as number of days increased and even at 4 months (Table 4). This behaviour of the RNA in our study could justify the reason for a weak negative correlation with time.

Table 4: Spectrophotometric analysis of absorbance ratio of nucleic acids

Time (hrs)	RNA Quantity (ng/µl)		RNA Absorbance ratio	
	1st loading	2nd loading	A 260/230	A 260/280
0 hrs	830.20	793.2	0.65	1.71
24 hrs	553.00	564.20	0.70	1.65
168 (7 days)	377.80	379.30	0.80	1.94
336 (14 days)	436.70	436.10	0.80	2.03
2880 (4 months)	563.80	553.20	0.70	1.66

A260/ 230 absorbance ratio indicates the presence of organic contaminants. Samples with 260/230 ratios below 1.8 are considered to have a significant amount of these contaminants that will interfere with downstream applications. This is especially true for reverse transcription. In a pure sample, the A260/230 should be close to 2.0. However, our study table shows significantly low A260/230 ratio revealing presence of contaminants in our samples.The A260/280 ratio is generally used to determine protein contamination of a nucleic acid sample. The aromatic proteins have a strong Ultra Violet absorbance at 280 nm. For pure RNA and DNA, A260/280 ratios should be somewhere around 2.1 and 1.8, respectively. An RNA purity of ratio of 1.8 -2.0 is accepted for many protocols. A lower ratio indicates the sample is protein contaminated. The presence of protein contamination may have an effect on downstream applications that use the nucleic acid samples. Thus we obtained pure RNA at day 7 and 14.

4. CONCLUSION

This study has proven that hair analysis can provide useful information in estimating post mortem interval as it revealed a correlation that exists between Post Mortem Interval and Nucleic acid degradation which can be used in ascertaining time of death. In forensic investigations requiring pure RNA for analysis, this research can be used as an inference to revealing that pure RNA can be obtained 7-14 days after death. In autopsy cases where time of death is known, it can be extracted to determine age of a biological evidence and as well in specimen identification.

ETHICAL APPROVAL

Ethical approval was granted by the Rivers State Ministry of Health with the number MH/PRS/391/VOL.2/800

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