REVIEW ARTICLE

Application of Ribonucleic Acid (RNA) in Forensic Sciences

Muhammad Jawad[1], Saadia Noreen[2], Muhammad Faheem Ashraf[3]
Maryam Asif[4], Hafiza Misbah Asif[5], Saqib Ali[6], Allah Rakha[7]

ABSTRACT

Forensic science has been helping law enforcement agencies in better understanding and presenting the evidence in the court of law. In certain situations, when conventional forensic methods of investigations cannot make better conclusions with more specific accuracy, then molecular techniques do help in reaching the acquired accuracy in the results regarding the identification of evidence. Advanced molecular techniques, which are using Deoxyribose Nucleic acid (DNA), Ribose Nucleic Acid (RNA), and protein molecules to produce forensically important information from the samples recovered at the crime scene. DNA can only distinguish among individuals but is unable to discriminate the type of samples originated from the same sample. For this RNA has become a molecule of interest for its different levels of expression in different cells/tissues of an individual. RNA molecules of different types are being used to build up models for several purposes (injury-age, new-born age, molecular cause of death, etc.). Modern techniques like Real-time Polymerase Chain Reaction (PCR) and Microarray are being used for the detection of RNA molecules of interest both in the form of its abundance and as a unique molecular detection. This script will help in understanding the importance of RNA application in forensic sciences by providing an overview of the research done to date and the techniques being used for this purpose.

KEYWORDS: Ribose nucleic acid, Genetics, Forensic, Molecular biology, Identification.

How to Cite This:

Jawad M, Noreen S, Ashraf MF, Asif M, Asif HM, Ali S, et al. Application of ribonucleic acid (RNA) in Forensic sciences. Biomedica. 2020; 36 (3): 232-47.

1. Muhammad Jawad
Department of Forensic Sciences, University of Health Sciences, Lahore – Pakistan.
2. Saadia Noreen
Department of Forensic Sciences, University of Health Sciences, Lahore – Pakistan.
3. Muhammad Faheem Ashraf
Department of Forensic Sciences, University of Health Sciences, Lahore – Pakistan.
4. Dr. Maryam Asif
Multan Medical and Dental College, Multan – Pakistan.
5. Dr. Hafiza Misbah Asif
Multan Medical and Dental College, Multan – Pakistan.
6. Saqib Ali
Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad – Pakistan.
7. Dr. Allah Rakha
Head, Department of Forensic Sciences, University of Health Sciences, Lahore – Pakistan.
Corresponding Author:
Dr. Allah Rakha
Head, Department of Forensic Sciences, University of Health Sciences, Lahore – Pakistan.
Email: dnatypist@gmail.com.

- Received for publication: 29-04-2020
- First revision received: 17-07-2020
- Second revision received: 04-09-2020
- Accepted for publication: 22-09-2020

INTRODUCTION

Forensic Science is helping law and order organizations by using various biological techniques. Deoxyribose nucleic acid (DNA) testing helps only in finding persons from the DNA obtained from the biological evidence.[1] Another under research, the molecular tool is the Ribose nucleic acid (RNA) testing, which has been proposed to help in recognizing the source of biological evidence as well as a substitute to several other forensic pathological tests such as to know death intervals, etc. To know the nature of biological evidence is a growing interest in the forensic science community.[1]

The data obtained from different molecular technologies have some meaningful applications. Molecular techniques are helping in generating Deoxyribose nucleic acid (DNA) profiles, tracking of molecular changes with the passage of time and identification of body fluids at the molecular level, etc. In forensic sciences that data is useful in declaring a person guilty or innocent, moreover, it also helps inthe identification of the deceased when no proper facial features are left after death. That is why with the emergence of molecular techniques, there is a surge in the development of forensic techniques.[2] One of the techniques is the use of biological material as evidence in the criminal investigation. After the start of the human genome project, forensic DNA testing came into existence, and protocols have been established, validated, and implemented in the field of forensic science.[2] However, in certain scenarios, e.g. sexual assault, the DNA testing may only help in correlating the accused person with the evidence obtained atthe crime scene, but it is unable to further deny the claims of the accused. The identification of the source, nature, and type of the evidence material that can further clarify the accusation and endorse the connection between the suspect and the evidence obtained can be reliably done through RNA testing.[3]

RNA-based testing is incorporating several types of RNA, some are used in the biosynthesis of protein, e.g. mRNA, tRNA, and rRNA, while other types (microRNA, anti-sense RNA, siRNA, and snRNA) play their role in regulating the genes at the post transcriptional level or acting as enzymes e.g. ribozymes.[4]

RNA testing is an important tool in solving several forensic cases like sexual assault, child abuse, finding death intervals, age of stains, and the source of the evidence like stains, chunk of meat, and 'touch DNA' the RNA testing has become a compulsory part of sexual assault cases in which a suspect person has vaginal intercourse with a female who was going through menses. Similarly, biological evidence in the form of blood is obtained from the suspect's clothes. The suspect argues that blood came from the nose of the victim, while the prosecution argues that the blood came in the result of a sexual act. Here it would become critical to know the source of the blood for validation of claims.[5] So the screenings of body fluids/tissues, blood, semen, saliva, vaginal secretion, menstrual blood and skin for micro RNA (miRNAs) is being possible through a whole full spectrum of micro RNA (miRNome) massively parallel sequencing approach.[6] In another example in which child abuse is involved and the accused person is one of the family members (thus having some legal proximity to the victim), and if some semen sample is recovered from the scene, which helps in identifying the suspect. Now, the accused person can challenge the DNA testing results on the basis that as he is a family member thus the biological evidence collected from the bed comes from the saliva (and not from the semen). In such a scenario the RNA-based testing would help in distinguishing the nature of biological evidence (either the recovered suspected body fluid was saliva or semen). Further, RNA can help us in providing valuable information, which is not possible directly from the genome (RNA degradation patterns for tracking the post-mortem changes, etc), this could be worth additional tool in examining post-mortem samples.[7] This review paper aims to provide an insight into the current literature and the future potential of RNA testing in the biological, technical, and forensic scenario.

Stability of RNA

The mRNA (with higher molecular weight) is more prone to degradation, while the miRNA is more stable due to their smaller size (~20 - 25 in length) and is more vulnerable to degradation caused by chemical and/or physical environmental strain, thus making it useful biomarker for body fluid identification.[8,9]

The mRNA has a wide range of stability. For example, the *c-fos*proto-oncogene transcript has a lifespan of 10-15 min, whereas the mRNA of globin remains stable for several hours.[10] This variability leads to the relative abundance of RNA that would be used for age determination. Anderson et al.[11] introduced the ratio of abundance between transcripts of two classes of RNA, namely, β-actin and 18S rRNA. Theoretically, 18S rRNA would degrade slowly as it is present in ribosomes and has an extra protecting layer of the ribosomes itself, whereas the B-actin mRNA would degrade faster due to less protection from the environment.[12]

miRNA for Forensic Application

The miRNA has been discovered as forensically relevant to biological fluids and has been investigated for body fluid identification in several forensic laboratories.[9] Lux et al.[13] reported the first case application of RNA-based tissue identification, in which they identified brain-specific RNA from the samples obtained from suicide or homicide used weapons.

Principle of Forensic RNA Testing

The DNA is present in all the body cells, while RNA being the intermediate part of the expression of hereditary material into protein, varies in body organs. The differentiation of cells involves the regulation of different genes, which means some genes do express transcriptionally while others remain dormant.[14] During a time span, each cell possesses a unique combination of the transcriptome that is unique for its presence and the relative abundance in each cell type.[15] This differential expression is known as 'gene expression signature' and is the key concept in identifying and or distinguishing a tissue/fluid.The other applications, i.e. finding death interval, involved the measurement of the elevated presence of a particular RNA.[16]

Isolation of RNA for Forensic Purpose

The first RNA application in forensic sciences has been reported back in 1984 and there has been extensive research on RNA application in forensic sciences. Juusola and Ballantyne proved in their early research that a sufficient quantity of mRNA can be extracted from the biological traces.[17],[18] Also, some studies showed that the co-extraction of DNA and RNA from the samples is possible, which results in a good amount of both of them for laboratory analysis.[19] Two different principles of co-extraction involve a commercial kit that uses a spin, mini-column methodology and a quick, simple nucleic acid isopropanol precipitation-based protocol. The simultaneous extraction of DNA and RNA via both methods results in a crucial quantity required for forensic case work which further helps in using a minute amount of sample for both DNA typing and detection of sources of forensically important biological material.[9,20-23] RNA extraction from different types of samples requires different protocols. For example, the protocol for RNA extraction from surgical bone tissues was suggested by Kuliwaba et al. [24] that the bone should be processed within 12 hours of retrieval for the reliable isolation of intact total RNA. Similarly, for RNA extraction from hair root for its being a useful source for genetic tests, Bradley et al.[25] reported a successful method for the detection of mRNA after ten days of storage at room temperature after plucking.

Techniques for RNA Detection

The methods being used for the detection of miRNA expression are northern blotting, microarray, and qPCR. Northern blot is nonspecific as it cannot discriminate between precursor and mature miRNA thus is unreliable. The throughput of miRNA profiling can be improved by using microarray, but it may not be employed due to low sensitivity and specificity along witha being costly. Hence quantitative polymerase chain reaction (qPCR) is of prime importance for nucleic acid detection due to its high sensitivity and specificity.[4] Eva Sauer et al.[26] worked on 15 preselected miRNAs by using the microarray technique and reported 5 miRNAs found to be organ tissue type-specific (Table-2).

Importance of Viability of RNA

The main problem in analyzing the crime scene stains is the degradation of RNA, which can lead to false-negative results.[27] RNA integrity can be maintained in minimizing the activity of RNase during the storage of samples and disruption. The integrity of the RNA presents meaningful biological data,[28] as the maximum RNA integrity is required for the procurement of the tissue samples.[29] Recovery of intact RNA from the deceased tissues is the major challenge as the integrity of RNA is compromised both by targeted and generalized ribonuclease enzymes (RNases) that degrade mRNA.[24] However, Lindenbergh et al.[30] used 28 years stored blood and semen stain samples for his study which indicates the long-term viability of the RNA. Some RNAs are more stable and tissue-specific thus is used as a reference marker.

Hemoglobin Subunit Beta (HBB) marker is a very stable mRNA and is highly specific for blood testing.[8] However, the accurate profiling of RNA depends upon the quality/purification of total RNA.[4]

The possibility of miRNAs as biomarkers for body fluid identification from pathological conditions requires the stability of RNA regardless of different cellular internal conditionsi.e. activity of ribonucleases or RNases.[31] There are two possibilities for the absence of a marker gene during detection either the missing of a particular type of fluid or the failed detection of severely degraded RNA. The possible solution for this problem is that a reference gene should be used as a positive control for each body fluid along with marker genes.[18] The housekeeping/or reference genes are expressed in certain body parts throughout the life span. The RNA from the genes recovered from the stains is a good source for the identification of the organs/fluids. Due to the degrading nature of RNA, there is a need for the use of more than one marker gene as the presence of one or all of them will decrease the ambiguity in identification.[32] However, an ideal reference gene must be equally or more stable than a transcript of interest.[18]

The co-extraction of RNA with DNA has become easy and time-saving. Now co-extracted RNA and DNA can be used for the molecular analysis either DNA or RNA-based. A study was conducted and validated by Haas et al.[22] by using co-extracted DNA and RNA. The results showed 'high sensitivity' in duplex and 'moderate sensitivity' in pentaplex amplification from co-extracted DNA and RNA.

The environmentally compromised biological stains can result in a better mRNA profiling and are therefore potentially useful for forensic casework analysis.[33] Although RNA-based testing has improved since the last few years,certain problems such as cross-reactivity with other species or tissues and specificity for particular tissues yet exist.[34] For degradation problem both quantitative (Qubit fluorometer and Nano Drop™) and qualitative tests such as denaturing gel electrophoresis and monitoring RNA integrity with RNA integrity number (RIN) is important to check for intact RNA. The typical values range from 1 to 10. 1 means RNA is degraded and 10 means RNA is intact, are necessary.[27]

Methods for RNA Analysis/Fluids Identification

RNA Analysis

Advanced methods being developed for mRNA profiling of forensic samples include capillary electrophoresis (CE)-based analysis, quantitative RT-PCR (qRT-PCR), and more recently, high-resolution melt (HRM) analysis and single-cell RNA sequencing.[15,35] Endpoint PCR capillary electrophoresis is an emerging technique for mRNA testing, it provides information regarding the presence or absence of a particular transcriptome. But the expression of fluid specific marker genes even in traces in other fluids may be a problem. Nussbaumer et al.[36] proved in his study the large amount presence of transcripts of haemoglobin alpha (HBA) locus 1 and mucein (MUC) in semen as well as in lower amounts of salvia and vaginal secretions. Thus, still there is a need for careful study and the validation of biomarkers to make sure that a particular expression is related to particular body fluid and there is no cross-reactivity.[18] The Endpoint PCR is well established and there is the easiness of data interpretation by just checking the presence or absence of a peak on the electrophoretogram. The capillary electrophoresis technique seems promising, for certain gene markers like kallikrein3 (KLK-3) that are only expressed in semen.[18]

Fleming et al. for the first time developed, validated and implemented mRNA multiplex RT-PCR for the detection of circulatory blood, menstrual blood, vaginal material, semen (with and without spermatozoa) and saliva. Latterly, Lindenbergh et al. showed a large-scale RT-PCR by using markers for each sample which include saliva, semen, menstrual secretion, vaginal mucosa, and skin cells.[32]

Conventional Methods for Body Fluids Identification

The conventional methods for forensic analysis including alternate light source, chemical tests (Luminol for blood detection), catalytic tests (Kastle-Meyer and benzidine tests for blood, and acid phosphatase test for semen detection), immunological tests (Hemastix for blood detection, and a prostate-specific antigen for semen detection). These conventional methods are presumption based and less confirmatory, one can

only presume the presence of a certain body fluid but cannot confirm the exact presence/type of the fluid. The methods involve the detection of different biological components (e.g. sugar, enzymes, and proteins, etc.).[37]The current forensic body fluid identification practices include chemical tests, immunological tests, protein catalytic activity tests (Luminol), spectroscopic methods (Raman spectroscopy), and microscopic examination of number and type of cells (e.g. from urine). Also, these methods can detect only one type of biological fluid stain while there is a shortage of samples and time. That's why it becomes vital to advent a method that can do testing for all types' fluids in a single step.[38]

The above mentioned conventional tests involve protein-protein interactions and/or chemical reactions for the detection of body fluids may be fast, inexpensive, easy to perform, and interpret for data analysis, but these tests may have some disadvantages e.g. in Kastle-Meyer test, a chemical being used for the detection of haemoglobin in trace evidence do have cross-activity with household chemicals (hypochlorite) as-well-as its no specific detection of haemoglobin from animal blood.[18,39] Takayama test is used for the presence of blood in the sample. The purpose of these tests is to narrow down the range of suspects or victims associated with a crime that is under investigation.[40] But there are certain demerits of these methods like consumption of sample, labor-intensive, time consuming, non-specific, and technologically diverse. To eliminate all such problems, there is a need for molecular-based identification of these fluids.

RNA-Based Body Fluids/Tissue Identifications

The main forensic application of the RNA is the identification of body fluids. It is important in terms of subsequent processing of samples in the lab.[18] A stain can be from different body fluids, e.g. blood, saliva, tears, sweat, urine, semen, menstrual blood, and vaginal material or the mixture of these fluids. The development of new biomarkers for fluids detection has been a matter of interest for forensic scientists.[41]

Erin K. Hanson et al.[42] discovered a panel of known miRNA—miR451, miR16, miR135b, miR10b, miR658, miR205, miR124a, miR372, and miR412— by using a minute quantity (50pg) of total RNA for the identification of forensically relevant body fluids.

Zheng et al.[9] reported three more miRNA markers for body fluid testing. They found that the relative expression ration (R) of different miRNA markers can distinguish between different body fluids. Currently, >1000 mature miRNA has been identified in the human genome.[4] Wang et al.[9] has worked on 754 known miRNA and found seven miRNA as fluid specific (miR16 and miR486 for venous blood, miR888, and miR891a for semen, miR214 formenstrual blood, miR124a for vaginal secretions, and miR138-2 for saliva).

Sirker et al.[43] identified five miRNA (miR10b, miR203, miR374, miR451, and miR943) by using ROC analysis for the identification of blood, saliva, semen, vaginal secretions, menstrual blood, and skin. Hanson et al.[42]found miRNA as inconsistent in their study while Wang et al.[4] used the mirVana™ miRNA isolation kit, which is particularly designed for the detection of siRNA and miRNA, and obtained consistent results.

Yan Xu et al.[44] developed a Highly Sensitive and Specific mRNA Multiplex System called (XCYR1) for forensic testing. They used 16 RNA markers for the identification of 10 different body fluids/tissue. For the first time, they used a marker gene for the identification of urine, sweat, and nasal secretions in a single multiplex PCR.

A multiplex system was developed by Song et al.[20] for body fluids identification. However, it was difficult to identify some samples found at the crime scene, especially body fluids mixtures, tissue mixtures, and traces samples, and emphasized on seeking more markers and methods to increase sensitivity and stability to obtain more reliable identification of a forensic stain are necessary. More recently, Dørum et al.[6] applied the whole miRNome massively parallel sequencing to six forensically relevant body fluids.

Blood Identification

Blood is the most prevalent evidence of violent crime scenes such as homicide and sexual assault.[40] It becomes a dire need to identify blood as body fluid and source of the blood. The identification of the source of the blood gains prime importance in

certain scenarios like sexual assaults. For example, in fraudulent cases where a victim can claim the bloodstains caused by menstruation blood as a result of injury and no other direct evidence is available to confirm the claim. To address such problems, RNA-based emerging research is being conducted.

In this perspective, several studies have been conducted to establish RNA-based identification of blood as a body fluid or source of the blood as either menstrual or venous.

Yan Xu et al.[44] were able to produce a full mRNA profile from a bloodstain on the wood. In another test, they used 2-year old blood stains and recovered the mRNA profile of one marker gene beta-globin (HBB) but amplification of DNA was unable to produce, which was presumed due to the possibility of a large amount of reticulocyte in the sample.[44] The other method for menstrual blood detectionis based on differences in isozyme patterns, fibrinolytic activity, hormone concentrations, and fibrin fibrinogen degradation products.[40]

Haas et al.[22] produced 'highly sensitive duplex' (HBB, HBA) and 'moderate sensitivity' pentaplex (ALAS2, CD3G, ANK1, PBGD, and SPTB).[22] Kohlmeier and Schneider[8] obtained successfully an RNA profile for two specific blood markers HBB, SPTB from 23-year blood stains from different career materials jeans, leather, wood, wallpaper, carpet, wool, and nylon fabric. Zubakov et al.[45] used the Affymetrix U133 plus2 Gene Chip platform for detecting the biomarkers for blood and saliva stains.

Skin Identification

"Every contact leaves a trace" (Locard's Principle, by Dr. Edmond Locard 1920). The skin identification is very important in forensic testing, for example, to corroborate the 'touch DNA' recovered from the evidence to the skin of the accused person. In manual strangulation cases, where victim and suspect were arguing while being in close proximity before killing the victim with his tie. The recovery of forensic DNA profile from the ligature (tie) may cause a doubt that the suspect's DNA was transferred via skin cells (fromthe suspect's hands) or form the saliva droplets, as they both were arguing. The suspect could claim his DNA transfer via saliva droplets rather he touched the tie to strangulate the victim, and that the victim accidentally strangled by his tie. In such cases, identification of the source of DNA (either saliva droplets or skin cells from hand)would be strong evidence against the suspect.[46] So, the mRNA-based test would be able to distinguish the source of evidence (which is the DNA of the suspect) either from saliva or skin cells.[46]

Song et al.[47] first time exploited the skin as a source of sample for investigating any correlation between mRNA andpost-mortem interval (PMI). They worked on four loci of two housekeeping genes *actinin alpha 1* (*ACTN1), actinin alpha 2 (ACTN2), Fattsyresyntes 1 (FASN1),* and *Fattsyresyntes 2 (FASN2*) as these have been used for time-dependent quantitative changes in mRNA.[47] Yan Xu et al.[44] used Cystatin-M er et protein (CST6) and Lorcirin protein(LOR)markers for skin identification, but they found a high cross-reactivity of these markers with other fluids, thus they eliminated these markers from their X-C Motif Chemokine Receptor 1) (XCYR1). Hanson et al.[46] worked on identifying the skin 'touch DNA' identification, he established multiplex RT-PCR comprising on five (Late Cornified Envelope 1C (LCE1C), Late Cornified Envelope 1D (LCE1D), Late Cornified Envelope 2D (LCE2D), C-C Motif Chemokine Ligand 27 (CCL27), Interleukin 37 (IL1F7) genes that are specific to the skin's shed cells. Recently, mRNA-based skin identification was performed on 11 candidate genes with quantitative PCR.[48]

Estimation of Postmortem Intervals (PMI)

The post-mortem interval is a duration elapsed from the time since death. The estimation of post-mortem intervals is of prime importance in forensic pathology as it helps with the identification of the human remains and contributes to investigating the possible causes of death.[49] The traditional methods of PMI are unreliable due to in accuracy as these methods involve the observation of physical modifications in the corps (algor, rigor, and livor mortis), which are affected by environmental factors (e.g. exposure of corps to water or heat).

The release of RNases both internal and exogenous is a major factor to be considered for estimating the PMI. The rapid release of lysosomal

RNases occurs in bones stored at 37ºC, and similarly, the release also occurred in when freezing and thawing of the samples is done.[24] The integrity and stability of the specific mRNA species is the function of noting the PMI.[24] The fragility of RNA could be exploited to help determine the post-mortem interval if there is a correlation between the quantity of residual RNA and elapsed time.[47] The estimation of PMI is affected by the rate of PM changes caused by different factors like environment temperature, body structure, and cause of death, location of the body, or putative drug consumption.[49]

A mathematical (mice) model was established by Sampaio-Silva et al.[49] to measure the PMI. They used five commonly used reference genes (Actb, Gapdh, Hprt, Cyp2E1, and Ppia) for their study and found that *Bhmt* and *Alb* genes specifically expressed in the liver and *Alb* is significantly correlated with PMI. They also found *Srp72* gene expression in the femoral quadriceps, and *Rps29* gene expression in all the tissue types (heart, liver, and femoral quadriceps) are strongly related to PMI, and thus can be pronounced as 'universal' biomarker for PMI estimation.[49]

Their findings of *Rps29* endorsed the results proposed by De Jonge et al.[50] to post-mortem samples. Scientists have observed no significant correlation between the mRNA human cardiac, brain, and skeletal muscle, this could be possible in case of using a smaller number of mRNA markers.[7,28,49]

Bauer et al.[23] found a strong correlation between mRNA degradation (from human blood and brain samples stored in the refrigerator for 5 days) and PMI. Koppelkamm et al.[28] observed the influence of various parameters like type of tissue, age at death, gender, and body mass index (BMI), as well as the duration of agony, cause of death, and post-mortem interval on the RNA integrity.

Wound Age Estimation

The determination of human dermal injury helps in forensic investigation. It can help the investigators to get answers to important questions such as the timing of the injury and incident the order of infliction (where there is more than one injury), the survival time after injury (post infliction interval), and the relationship of the injury to the incident. A lot of research is being done to develop some methods for the accurateestimation of human dermal injury age.[51]

Wound estimation has always been a challenge in forensic pathology. With the rapid development of bioinformatics and molecular biology-based detection technology, the contemporary trend is to infer time using DNA or RNA.[52] Qiu-xiang et al.[52] worked on mRNA expression levels of seven candidate genes (ICAM-1, NF-κB, MX2, MT1, MT2, and Cox6c) and expression trends of the seven genes were all found to be related to wound age. Palagummi et al.[51] developed an RNA-based multiplex assay for the simultaneous investigations of mRNA expression in wounds. They observed the mRNA expression patterns CMA1, DUSP1, IL1b, IL7, TNFa, and VEGFA genes having potential in estimating human dermal injury age, but only at earlystages (days) of injury healing.

Age Determination of Biological Evidence Trace/stains

The determination of the time of deposition of biological evidence is the most intriguing thing in forensic science. For example, the recovery of a hair on the crime scene becomes very interesting that either it was left there during the crime was committed or its presence before the crime and no immediate connections. The age determination is based on the degradation time of RNA. The first attempt was made by Bauer et al.[23] as they studied the pattern of degrading mRNA changes. Anderson et al.[11] found the ratio between β-actin and 18S rRNA successful in predicting the age of the stain. However, for stain age determination, it is better to compare transcripts from different RNA classes instead of comparing transcripts of the same RNA class.

Molecular Analysis of Cause of Death

Investigating the cause of human death is of prime importance in forensic science. The conventional postpartum examination is not just enough to explain the cause of a person's death, but there is a dire need for further molecular testing.[18]

Death of a body does not mean the cessation of cell processors and the variation in gene expression patterns. A death without any prominent physical

signs on the dead body may be a natural death or the result of the killing. The killing may be done in several ways, e.g. via mechanical asphyxiation, poison, hitting (without any sign on the body), and drug abuse. In all these cases, there is a need for the development of molecular-based testing. Until now some studies have been done on rats.

The examination of gene expression levels during post-mortem analysis of cause or circumstance during death may lead to some novel application of molecular biology in forensics.[27] The isolation of RNA after several days of post-mortem is a breakthrough in utilizing the RNA for a death investigation. [53,54] The main problem of the use of RNA in post-mortem testing in the short time stability of RNA, but several researchers have reported the recovery of a commendable quantity of RNA from dead bodies after several days.[54,55] Zhao et al.[56] possibly presented the first study of the biomarkers for the determination of the cause of death. Ikematsu et al.[57] successfully identified the conditional change of transcriptome after the death of rats. Also, Zhao et al.[56] studied the changes in expression levels before and after the death of a human. The change in expression in vascular endothelial growth factor (VEGF) strongly differentiate the cardiac death versus asphyxia.[56]

Drug Abuse

Matsuo et al.[58] studied the change in expression in rats by exploiting them to methamphetamine. Martin et al. confirmed the changes in the expression of c-fos, fosB, Crf, Cck, and Npas4 transcripts due to the exposure of non-toxic methamphetamine injections to the rates.[59] Similarly, Jean Lud Cadet et al.[60] worked on the methamphetamine-induced dopamine-independent alterations in striatal gene expression in the 6- hydroxydopamine hemi parkinsonian rats. There is a dire need to check the discovery of genetic response to the use of different drugs, and the establishment of RNA-based testing for different drug abuses.[61]

Sex Determination

The sex determination of a required person becomes crucial to the investigators when biological evidence is recovered from the scene.[62] DNA-based sex determination is being done (amplification of amelogenin), however, RNA-based discrimination is being researched. Puoti et al.[63] investigated the RNA for sex determination in *Caenorhabditis elegans.* Berge et al.[3] studied the sex-specific RNA markers and worked on multiplexes to simultaneously identify the sex of the donor and the cell type of the material. Silva et al.[31] identified a subset of four miRNAs (miR-548-3p, miR-1323, miR-940, and miR-1292) to be highly regulated in females (63–95%), demonstrating its potential to distinguish gender. Similarly, Wang et al.[64] showed that mir-130b and mir-18b had slightly higher concentrations in male serum samples as compared to females. Another study attested a significant change in two miRNAs, let-7 g and miR-221, which were overexpressed in the serum of individuals and more prominent in women.[65]

Tissue origin of Stains

The identification of organ tissues becomes important if the biological traces are recovered from the scene, which may help in forensic investigations. Traditional identification techniques like histological and immunological assays would be replaced with molecular genetic approaches that could result in superior to them.[26]

The new emerging molecular biological tools would require a minor amount of biological evidence material (stain) to test its nature or its source.[34] The biomarkers used for stain identification would be specific for a particular stain as well as having an ability of survival from environmental insults that a forensic sample may encounter. Conti and Buel[34] extracted RNA from samples of age up to 4 years. Conti and Buel amplified semen specific gene PRM2 from 1666 days old seminal stain stored at room temperature in the dark. They also noted no big difference in Cts from samples exposed long (>500 days) period to elevated temperature conditions. HBB gene is very stable and can be detected from 13 to 26 months old blood stains.[34] Similarly, the PRM2 gene is also very stable and can be detected from 16 to 24 months old seminal stains. Moreover, mRNA profiling assay for organ tissue identification using a targeted panel of 46 mRNA biomarkers for tissue, organ identification is effective.[66]

Identification of New-born's Age

Age identification becomes very important in certain cases like suicide terrorist attacks and child kidnapping. New-born identification is useful in certain crimes as child kidnapping, assault, criminal abortion investigation, and hospital abduction. In criminal abortion cases, it becomes important to distinguish between the new-born blood and adult blood.[67]

In theory, a comparison of the gene expression profile from individuals of different ages could reveal the constellations of candidate genes whose expression is correlated with a specific age.[67] The discovery of age-specific expression of different genes would help in determining the age. A typical example of age-specific gene expression is haemoglobin gene switching.[68] As human β-haemoglobin locus present on chromosome number 11 encodes five β-like haemoglobin ε, $^{G}\gamma$, $^{A}\gamma$, δ and β, and β –pseudogene ($^{\beta}\psi$).[69] There is a shift in the expression of these genes with the passage of life. Alvarez and Ballantyne[67] first time identified two novel gamma haemoglobin transcripts (HBG1n and HBG2n) that exhibit restricted expression in the blood of (human) new-born children. MiRNAomes and DNA methylomes in the same CD4+T cell roles were demonstrated in individuals during the aging process.[70]

Table-1: Comparison between conventional and RNA-based methods.[5,71,72]

Conventional Method	RNA-based Method
• Can test a stain only for one type of body fluid at a time. • These are presumptive and qualitative tests • Nonspecific (cross-reactive with other species) for example detection of saliva by detecting enzyme amylase that is also present in other fluids (pancreatic, urinary) [34] • No test for menstrual blood and vaginal secretions • Require multiple tests for fluid identification (first presumptive test for the detection of fluid then confirmatory test for the origin of fluid) • Technologically diverse techniques • Available for only some fluid types (semen, blood) • Costly in terms of: - Time (more steps) - Labor required (technical person, histologist, serologist, etc) - Sample consumption • For example: - Takayama test, immunological identification for blood [74] - Immunological sperm detection by using prostate-specific antigen (PSA or p30) - Blood stains are tested via tetrabase (4,4-bis (dimethylamino) diphenylmethane) test [75], the Kastle–Meyer phenolphthalein test, the tetramethylbenzidine test [76], the orthotolidine test [77], or the luminol (3-aminophthalhydrazide) chemoluminescence test - Phadebas test is for the detection of saliva via an enzymatic amylase test[78]	• Multiplex ability can analyze all the possible types of body fluids. • These are confirmatory and quantitative tests • mRNA based biomarkers have been identified to distinguish between menstrual blood and vaginal secretions • Can perform both works in one step/test. • Can detect all types of body fluids (semen, blood, sweat, urine, saliva or vaginal, etc.) • Technologically same techniques • Detection of several markers at a time • Economical in terms of: - Time (fewer steps) - Labor required (only one technician, i.e. serologist can do all steps) - Sample consumption (the ability of co-extraction with DNA[73]) • Tissues specific mRNA, miRNA, snRNA test

Table-2: RNA markers used during research for different purposes.

Body Fluid	RNA Markers	References
Circulatory Blood Blood Stains	Delta-aminolevulinate synthase (ALAS2)	22, 37, 41, 71
	Haemoglobin alpha (HBA)	22, 36, 41
	Haemoglobin beta (HBB)	22, 34, 41
	Beta-spectrin (SPTB)	22, 41, 67, 33, 34, 71
	Porphobilinogen deaminase (PBGD)	22, 33, 41, 71
	CD3 $_{\gamma}$ molecule (CD3G)	22, 34, 41
	Ankyrin 1 (ANK1)	22, 41, 79
	Glycophorin A (GLY)	44
	CASP2, C1QR1, ALOX5AP, AQP9, C5R1, NCF2, MNDA, ARHGAP26	45
	AM1CA1	30, 45

	GYPA	80
	Pro-platelet basic protein (PPBP)	21
	ALAS2, ANK1, HBB	15
	SPTB, PBGD	33
	HBA, ALAS2, GlycoA	20
	miR-190a-5p, miR-454-3p	81
	HBD, SLC4A1	82
	miR451	43
	hsa-miR-144-3p	83
	CASP1, AMICA1, C1QR1, ALOX5AP, AQP9, C5R1, NCF2, MNDA, ARHGAP26	45
	Matrix metalloproteinase 7 (MMP7)	23, 37, 41, 71
	Matrix metalloproteinase 10 (MMP10)	23, 71, 79
	Matrix metalloproteinase 11 (MMP11)	23, 41, 80
	Glycophorin A (GlycoA)	80
	Keratin 19 (CK19), Progesterone Receptor (PR)	5, 71, 80
Menstrual Blood	NCF2, MNDA, CASP1, C1QR1, ARHGAP26, AMICA1, ALOX5AP, AQP9, C5R1	84
	LEFTY2, MMP10	15
	HBA, ALAS2, GlycoA, HBD1, MUC4, Lcris, Lgas, MMP7, MMP10, MP11	20
	miR-141-3p, miR-497-5p	81
	MMP3, STC1	82
	miR16	43
	miR-412-3p	85
	Statherin (STATH)	5, 33, 37, 41, 71, 80
	Histatin 3 (HTN3)	5, 33, 37, 41, 67, 71, 80
	PRB4	79
	SPRR3, SPRR1A, KRT6A	45
Saliva	KRT4, KRT13	30, 45
	Follicular dendritic cell secreted protein (FDSCP)	21
	HTN3, MUC7, STATH	15
	STATH, HTN3	33
	miR-935, miR-3168	81
	SPRR3, SPRR1A, KRT4, KRT6A, and KRT13	45
Saliva Stains	KRT4, KRT6A, KRT13, SPRR1A, SPRR2A	84
	STATH, HTN3, MUC7	20
Nasal Secretions	Statherin (STATH)	44
	Protamine 1 (PRM1)	23, 33, 41, 67, 71
	Protamine 2 (PRM2)	23, 33, 34, 37, 41, 71, 80
	KLK/prostate-specific antigen (PSA)	34, 36, 41
	Transglutaminase 4 (TGM4)	37, 79, 80
	Semenolegin 1 (SEMG1)	34, 41, 79
	SEMG1	79
	MSP	79
Semen	Microseminoprotein (MSMB)	21
	PRM2, SEMG1, TGM4	15
	PRM1, PRM2	33
	PRM2, SEMG1, TGM4	20
	miR-888-5p, miR-135a-5p	81
	TNP1, KLK2	82
	miR10b, miR374	43
	miR-10b-5p	85
	Human β-defensin 1 (HBD1)	71
	Mucin 4 (MUC4)	33, 36, 37, 41, 67, 71
	16S rRNA	86
Vaginal Secretions	Human β-defensin 1 (HBD1)	41, 71
	ESR1	79
	SFTA2, FUT6, DKK4, IL19, MYOZ1, CYP2B7P1	46
	Mesothelin (MSLN)	21

	CYP2A7, CYP2B7P1, DKK4, FUT6, IL19, MYOZ1, NOXO1	15
	MUC4	33
	HBD1, MUC4, Lcris, Lgas	20
	miR-3134, miR-31-3p	81
	miR1280, miR4286, miR124, miR203, miR205	43
	miR-124a-3p	85
	Corneodesmosin (CDSN)	48
	Loricrin (LOR)	48
	Cytokeratin 9 (KRT9)	48
	LCE1D, LCE2D, CCL27, IL1F7, LCE1C	46
Skin/touch-DNA	CST6	44
	CDSN, LOR, KRT9	48
	CDSN, CST6, DSC1	87
	CCL27, IL1F7, KRT9, LCE1C, LCE2D	15
	miR-4761-5p, miR-137, miR-4473, miR-585-3	81
	miR3169	43
Touch-DNA	LCE1C, LCE1D, LCE2D, CCL27, IL1F7	46
Oral Mucosa	Keratin 4 (KRT4)	44
Urine	Uromodulin (UMOD)	44
Sweat	DCD	44
Brain	C1orf61	13
Sex Determination from Biological Samples Parts	XIST for Female RPS4Y1 for Male	3
Brain	hsa-miR-219a-5p	26
Liver	hsa-miR-122-5p	26
Skin	hsa-miR-205-5p	26
Heart	hsa-miR-208b-3p	26
Skeletal	hsa-miR-206	26
Drug Abuse	c-fos, fosb, c-jun, junB, Crf, Nmu, Cck, Npas4	59
	Tac1, Tac2, Pdyn, Nts, Nmu, Fst. Inhba, Acvr1, Pdgf-d, Cox-2, Syt, Synj2bp	60
	ICAM-1, NF-κB, MX2, MT1, MT2, sTnI, Cox6c	52
Wound Estimation	(CMA1), (COL3A1), (DUSP1), (IL1b), (IL2), (IL6), (IL7), (TNFa), (VEGFA)	51
	Fosb, Atf3, IL6, Cxcl1, Zfp36, Jun, Rasd1, Fos, Apold1, Sfrp2, Fcna,	88
	GAPDH, 18SrRNA, S15, beta-actin	5, 21, 28, 44
Positive Control (Housekeeping gene)	B2M, COX1, HPRT1, PGK1, PPIH, S15, TCEA1, TFRC, UBC, UBE2D2	15
	S15, β-actin, GAPDH	33

Technologies Being Used

Nano String Technology

To make an authentic identification of tissues, there is a dire need for the use of more than one marker. For this a new technique, Nano String technology was used by Jong-Lyul Park et al. [39] for the identification of multiple forensic markers. The Nano Stringn Counter (Nano String Technologies, Seattle, WA, USA) is a recent platform that can quantify the expression of hundreds of mRNAs in a single reaction using color-coded molecular barcodes.[89] Jong-Lyul Park et al.[39] used 18 body fluid-specific mRNA markers at the same time. They used R software (version 2.6.1) for the data interpretation and $P > 0.5$ was considered more accurate in their body fluid relation.

Real – Time PCR

The RT-PCR allows the detection of relative gene expression levels in different samples.[40] Quantitative reverse transcription PCR (qRT-PCR) is very sensitive and specific as compared to the other two precedent approaches and can be used for absolute quantification. In forensic cases, the quantity of genetic material collected can be less, and therefore, it is important to use a methodology that requires a fairly low amount of RNA. qRT-PCR beyond being a well-established method, it also requires a low amount of RNA and has a low cost per sample. On the other hand, it also presents a significant disadvantage, it cannot identify novel miRNAs.[31]

End Point PCR Capillary Electrophoresis

Endpoint PCR allows the detection of a transcript only if it is available in enough quantity. The main problem during this technique is the cross-reactivity of markers.

Microarray

Microarray technology can study the expression of thousands of genes at once involving a low-cost price. The easiness of analyzing the expression of all the genes at a time through microarray makes the scientists be able to detect the body fluid. Both DNA and RNA can be used for microarray analysis. But due to its lack of specificity and sensitivity as compared to real-time PCR or RNA sequencing, the microarray is considered as a real disadvantage that can tamper the results. Moreover, a large amount of sample is required for experimentation, which is a major problem with forensic samples.[31] Park et al.[21] used the Affymetrix U133 Plus2 platform to find out the fluid specific mRNA biomarkers, and selected 137 genes from microarray gene expression profiling. Lou et al.[81] performed genome-wide miRNA expression profiling by using Exiqon'smiRCURYTM LNA Array technology to screen out novel miRNA markers for body fluids. Lee et al.[29] used Affymetrix gene chip for a comparison study on post-mortem samples on the mouse and repeated the same method for human., and found that Affymetrix gene chip can detect degraded RNA, as its unique detection from the 3` end of transcripts.[29]

Similarly, Albani et al.[82] characterized six novel mRNA for body fluids by using the Massively parallel sequencing (MPS)technique for the first time to search novel body fluid RNA markers.

High-Resolution Melt (HRM) Analysis

To simplify and reduce the time and cost of RNA analysis, Erin K. Hanson et al.[90] used another technology known as High-Resolution Melt. This is a bio-analytical method being used for finding the mutation in a DNA sequence. This ideology of this technique is the identification of PCR products (amplicons) by their melting temperatures. The melting temperature of an amplicon is dependent on the length of its sequence and the ionic strength of its environment, which can be measured post-PCR.

It can be used in forensics for tissue/fluid identification by measuring the specific amplicons of body fluids/tissue. It has several advantages over other methods. For example, it saves both cost and time. Other methods like EC, RT-PCR requires costly labelled primers for the detection of signals, while this method uses unlabelled primers. EC involves separate steps for amplification and detection, thus increasing the time of analysis. HRM analysis takes almost the same time as by RT-PCR (2hours) but being multiplex is the advantage of HRM over RT-PCR.[90]

CONCLUSION

The conventional methods being used in forensic sciences for different purposes have been known to show promising results. But in the modern era of emerging technology and flourishing molecular research, Ribonucleic Acid (RNA) is an emerging molecule of interest to replace the conventional methodologies, for its being more authentic and having high sensitivity and specificity.

The RNA-profiling multiplex technique shows a promising method to differentiate body fluids in mixed ratios To analyze an unknown body fluid mixture, it is necessary and meaningful to first use an RNA-profiling. Similarly, other applications of RNA in forensic sciences are emerging, e.g. cause of death, age at death, gender and body mass index, duration of agony, and storage conditions of the body.Moreover, RNA-based gender identification from body fluids will also be developed.

FUTURE PERSPECTIVE

In near future, there is a potential for the development of RNA-based markers which would help in investigating the type of circumstances before a death occur, for example, if a person is found dead and physically it sounds a suicide, but that person may be under certain pressure and there would be some genes which expressed under pressure/tension situation.

LIMITATIONS OF THE STUDY

Every day, a huge volume of new research is being

added. An attempt was made to cover maximum information regarding RNA application in forensic sciences. However, more literature is needed to shed detailed light on the emerging RNA-based forensic tool.

ACKNOWLEDGEMENT

The authors would like to acknowledge University of Health Sciences Lahore for HEC digital library for free access to articles.

CONFLICT OF INTEREST

None to declare.

GRANT SUPPORT & FINANCIAL DISCLOSURE

None to disclose.

REFERENCES

1. Gill P, Hicks T, Butler JM. DNA commission of the International society for forensic genetics: Assessing the value of forensic biological evidence-Guidelines highlighting the importance of propositions: Part I: evaluation of DNA profiling comparisons given (sub-source propositions. Forensic Sci Int-Gen. 2018; 36 (2): 189-202.
2. Sijen T. Molecular approaches for forensic cell type identification: on mRNA, miRNA, DNA methylation and microbial markers. Forensic Sci Int-Gen. 2015; 18 (5): 21-32.
3. van den Berge M, Sijen T. A male and female RNA marker to infer sex in forensic analysis. Forensic Sci Int-Gen. 2017; 26 (4): 70-6.
4. Wang Z, Luo H, Pan X, Liao M, Hou Y. A model for data analysis of microRNA expression in forensic body fluid identification. Forensic Sci Int-Gen. 2012; 6 (3): 419-23.
5. Juusola J, Ballantyne J. The development of an RNA based assay system to supplant conventional methods for body fluid identification. AAFS. 2002.
6. Dørum G, Ingold S, Hanson E, Ballantyne J, Russo G, Aluri S, et al. Predicting the origin of stains from whole miRNome massively parallel sequencing data. Forensic Sci Int-Gen. 2019; 40(3): 131-9.
7. Fordyce SL, Kampmann M-L, Van Doorn NL, Gilbert MTP. Long-term RNA persistence in postmortem contexts. Investig Genet. 2013; 4 (1):1-7.
8. Kohlmeier F, Schneider PM. Successful mRNA profiling of 23 years old blood stains. Forensic Sci Int-Gen. 2012; 6 (2): 274-6.
9. Wang Z, Zhang J, Luo H, Ye Y, Yan J, Hou Y. Screening and confirmation of microRNA markers for forensic body fluid identification. Forensic Sci Int-Gen. 2013; 7 (1): 116-23.
10. Chen CYA, Ezzeddine N, Shyu AB. Messenger RNA half-life measurements in mammalian cells. Method Enzymol. 2008; 448(1): 335-57.
11. Anderson S, Howard B, Hobbs GR, Bishop CP. A method for determining the age of a blood stain. Forensic Sci Int. 2005; 148 (1): 37-45.
12. Shoshani O, Cleveland DW. Gene expression regulated by RNA stability. Science. 2020; 367 (6473): 29-32.
13. Lux C, Schyma C, Madea B, Courts C. Identification of gunshots to the head by detection of RNA in backspatter primarily expressed in brain tissue. Forensic Sci Int. 2014; 237(4): 62-9.
14. Shewale JG, Liu RH. Forensic DNA analysis: current practices and emerging technologies. CRC Press; 2013.
15. Danaher P, White RL, Hanson EK, Ballantyne J. Facile semi-automated forensic body fluid identification by multiplex solution hybridization of Nano String® barcode probes to specific mRNA targets. Forensic Sci Int-Gen. 2015; 14(1): 18-30.
16. Wang S, Wang Z, Tao R, He G, Liu J, Li C, et al. The potential use of Piwi-interacting RNA biomarkers in forensic body fluid identification: a proof-of-principle study. Forensic Sci Int-Gen. 2019; 39(2): 129-35.
17. Buckley L. RNA-DNA ratio: an index of larval fish growth in the sea. Mar Biol. 1984; 80 (3): 291-8.
18. Mallett X, Blythe T, Berry R. *Advances in forensic human identification.* CRC Press; 2014.
19. Loureiro S, Amorim A, Cainé L, Silva B, Gomes I. Evaluation of two DNA/RNA co-extraction methods for body fluid identification in forensics. Forensic Sci Int-Gen. 2019;7(1):250-2.
20. Song F, Luo H, Hou Y. Developed and evaluated a multiplex mRNA profiling system for body fluid identification in Chinese Han population. J Forensic Leg Med. 2015; 35(7): 73-80.
21. Park SM, Park SY, Kim JH, Kang TW, Park JL, Woo KM, et al. Genome-wide mRNA profiling and multiplex quantitative RT-PCR for forensic body fluid identification. Forensic Sci Int-Gen. 2013; 7 (1): 143-50.
22. Haas C, Hanson E, Anjos MJ, Bär W, Banemann R, Berti A, et al. RNA/DNA co-analysis from blood stains—results of a second collaborative EDNAP exercise. Forensic Sci Int-Gen. 2012; 6 (1): 70-80.

23. Bauer M, Gramlich I, Polzin S, Patzelt D. Quantification of mRNA degradation as possible indicator of postmortem interval—a pilot study. Leg Med. 2003; 5 (4): 220-7.
24. Kuliwaba JS, Fazzalari NL, Findlay DM. Stability of RNA isolated from human trabecular bone at post-mortem and surgery. BBA-Mol Basis Dis. 2005; 1740 (1): 1-11.
25. Bradley BJ, Pastorini J, Mundy NI. Successful retrieval of mRNA from hair follicles stored at room temperature: implications for studying gene expression in wild mammals. Mol Ecol Notes. 2005; 5 (4): 961-4.
26. Sauer E, Extra A, Cachée P, Courts C. Identification of organ tissue types and skin from forensic samples by microRNA expression analysis. Forensic Sci Int-Gen. 2017; 28(3): 99-110.
27. Vennemann M, Koppelkamm A. mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Sci Int. 2010; 203 (1-3): 71-5.
28. Koppelkamm A, Vennemann B, Lutz-Bonengel S, Fracasso T, Vennemann M. RNA integrity in post-mortem samples: influencing parameters and implications on RT-qPCR assays. Int J Legal Med. 2011; 125 (4): 573-80.
29. Lee J, Hever A, Willhite D, Zlotnik A, Hevezi P. Effects of RNA degradation on gene expression analysis of human postmortem tissues. Faseb J. 2005; 19 (10): 1356-8.
30. Lindenbergh A, de Pagter M, Ramdayal G, Visser M, Zubakov D, Kayser M, et al. A multiplex (m) RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int-Gen. 2012; 6 (5): 565-77.
31. Silva SS, Lopes C, Teixeira A, de Sousa MC, Medeiros R. Forensic miRNA: potential biomarker for body fluids? Forensic Sci Int-Gen. 2015; 14(2): 1-10.
32. Lindenbergh A, Maaskant P, Sijen T. Implementation of RNA profiling in forensic casework. Forensic Sci Int-Gen. 2013; 7 (1): 159-66.
33. Setzer M, Juusola J, Ballantyne J. Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci. 2008; 53 (2): 296-305.
34. Conti T, Buel E. Forensic stain identification by RT-PCR analysis. Biblio Gov; 2012.
35. Chen G, Shi T. Single-cell RNA-seq technologies and related computational data analysis. Front Genet. 2019; 10(1): 317-22.
36. Nussbaumer C, Gharehbaghi-Schnell E, Korschineck I. Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. Forensic Sci Int. 2006; 157 (2-3): 181-6.
37. Richard MLL, Harper KA, Craig RL, Onorato AJ, Robertson JM, Donfack J. Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis. Forensic Sci Int-Gen. 2012; 6 (4): 452-60.
38. An JH, Shin K-J, Yang WI, Lee HY. Body fluid identification in forensics. 2012;45(10): 545-53.
39. Park JL, Park SM, Kim JH, Lee HC, Lee SH, Woo KM, et al. Forensic body fluid identification by analysis of multiple RNA markers using NanoString technology. Genomics Inform. 2013; 11 (4): 277-81.
40. Counsil TI, McKillip JL. Forensic blood evidence analysis using RNA targets and novel molecular tools. Biologia. 2010; 65 (2): 175-82.
41. Haas C, Hanson E, Bär W, Banemann R, Bento AM, Berti A, et al. mRNA profiling for the identification of blood—results of a collaborative EDNAP exercise. Forensic Sci Int-Gen. 2011; 5 (1): 21-6.
42. Hanson EK, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem. 2009; 387 (2): 303-14.
43. Sirker M, Fimmers R, Schneider P, Gomes I. Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Sci Int-Gen. 2017; 27(2): 41-9.
44. Xu Y, Xie J, Cao Y, Zhou H, Ping Y, Chen L, et al. Development of highly sensitive and specific mRNA multiplex system (XCYR1) for forensic human body fluids and tissues identification. PloS one. 2014; 9 (7): e100123.
45. Zubakov D, Hanekamp E, Kokshoorn M, van IJcken W, Kayser M. Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med. 2008; 122 (2): 135-42.
46. Hanson E, Haas C, Jucker R, Ballantyne J. Specific and sensitive mRNA biomarkers for the identification of skin in 'touch DNA'evidence. Forensic Sci Int-Gen. 2012; 6 (5): 548-58.
47. Song HJ, Choi YL, Kang SY, Chae JM. The effect of elapsed time on the quantity of mRNA in skin: a study to evaluate the potential forensic use of mRNA to determine the postmortem interval. Korean J Leg Med. 2012; 36 (2): 151-8.
48. Visser M, Zubakov D, Ballantyne KN, Kayser M. mRNA-based skin identification for forensic applications. Int J Legal Med. 2011; 125 (2): 253-63.
49. Sampaio-Silva F, Magalhães T, Carvalho F, Dinis-Oliveira RJ, Silvestre R. Profiling of RNA degradation for estimation of post morterm interval. PloS one. 2013; 8 (2): e56507.

50. De Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, et al. Evidence based selection of housekeeping genes. PloS one. 2007; 2 (9): e898.
51. Palagummi S, Harbison S, Elliot D, Fleming R. A multiplex analysis of RNA expression during injury healing in human dermal injuries for injury age estimation. Forensic Sci Int Genet Suppl Ser. 2013; 4 (1): e17-8.
52. QiuFxiang D, XiaoKwei W, Lei Z, SanSqiang L, CaiSrong G, YingS W. Relative Expression of Indicators for Wound Age Estimation in Forensic Pathology. J Forensic Med. 2015; 31 (2):81-2.
53. Catts VS, Catts SV, Fernandez HR, Taylor JM, Coulson EJ, Lutze-Mann LH. A microarray study of post-mortem mRNA degradation in mouse brain tissue. Mol Brain Res. 2005; 138 (2): 164-77.
54. Heinrich M, Matt K, Lutz-Bonengel S, Schmidt U. Successful RNA extraction from various human postmortem tissues. Int J Legal Med. 2007; 121 (2): 136-42.
55. Preece P, Virley DJ, Costandi M, Coombes R, Moss SJ, Mudge AW, et al. An optimistic view for quantifying mRNA in post-mortem human brain. Mol Brain Res. 2003; 116 (1-2): 7-16.
56. Zhao D, Ishikawa T, Quan L, Li DR, Michiue T, Yoshida C, et al. Tissue-specific differences in mRNA quantification of glucose transporter 1 and vascular endothelial growth factor with special regard to death investigations of fatal injuries. Forensic Sci Int. 2008; 177 (2-3): 176-83.
57. Ikematsu K, Tsuda R, Nakasono I. Gene response of mouse skin to pressure injury in the neck region. Leg Med. 2006; 8 (2): 128-31.
58. Matsuo A, Ikematsu K, Nakasono I. C-fos, fos-B, c-jun and dusp-1 expression in the mouse heart after single and repeated methamphetamine administration. Leg Med. 2009; 11 (6): 285-90.
59. Martin TA, Jayanthi S, McCoy MT, Brannock C, Ladenheim B, Garrett T, et al. Methamphetamine causes differential alterations in gene expression and patterns of histone acetylation/hypoacetylation in the rat nucleus accumbens. PloS one. 2012; 7 (3): e34236.
60. Cadet JL, Brannock C, Krasnova IN, Ladenheim B, McCoy MT, Chou J, et al. Methamphetamine-induced dopamine-independent alterations in striatal gene expression in the 6-hydroxydopamine hemiparkinsonian rats. PLoS One. 2010; 5 (12). e15643.
61. Bu Q, Long H, Shao X, Gu H, Kong J, Luo L, et al. Cocaine induces differential circular RNA expression in striatum. Transl Psychiatry. 2019; 9 (1): 1-11.
62. Sabarinath B. Age and sex determination. J Forensic Dent Sci. 2011; 3 (1): 46-7.
63. Puoti A, Pugnale P, Belfiore M, Schläppi AC, Saudan Z. RNA and sex determination in Caenorhabditis elegans. EMBO Rep. 2001; 2 (10): 899-904.
64. Wang K, Yuan Y, Cho J-H, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. PloS one. 2012; 7 (7):e41561.
65. Teixeira SA, Ibelli AM, Cantão ME, Oliveira HC, Ledur MC, Peixoto JD, et al. Sex Determination Using RNA-Sequencing Analyses in Early Prenatal Pig Development. Gene. 2019; 10 (12): 1010.
66. Hanson E, Ballantyne J. Human organ tissue identification by targeted RNA deep sequencing to aid the investigation of traumatic injury. Gene. 2017; 8 (11): 319-41.
67. Alvarez M, Ballantyne J. The identification of newborns using messenger RNA profiling analysis. Anal Biochem. 2006; 357 (1): 21-34.
68. Jane SM, Cunningham JM. Molecular mechanisms of hemoglobin switching. Int J Biochem Cell Biol. 1996; 28 (11): 1197-209.
69. Martin DI, Fiering S, Groudine M. Regulation of β-globin gene expression: straightening out the locus. Curr Opin Genet Dev. 1996; 6 (4): 488-95.
70. Zhao M, Qin J, Yin H, Tan Y, Liao W, Liu Q, et al. Distinct epigenomes in CD4+ T cells of newborns, middle-ages and centenarians. Sci Rep. 2016; 6: 38411.
71. Juusola J, Ballantyne J. Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int. 2005; 152 (1): 1-12.
72. Juusola J, Ballantyne J. Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int. 2003; 135 (2): 85-96.
73. Patel G, Peel C. Identifying the origin of cells. Forensic Sci Int Genet Suppl Ser. 2008; 1 (1): 574-6.
74. Hochmeister MN, Budowle B, Sparkes R, Rudin O, Gehrig C, Thali M, et al. Validation studies of an immunochromatographic 1-step test for the forensic identification of human blood. J Forensic Sci. 1999; 44 (3): 597-602.
75. Lomholt B, Keiding N. Tetrabase, an alternative to benzidine and orthotolidine for detection of haemoglobin in urine. Lancet. 1977; 309 (8011): 608-9.
76. Garner D, Cano K, Peimer R, Yeshion T. An evaluation of tetramethylbenzidine as a presumptive test for blood. J Forensic Sci. 1976; 21 (4): 816-21.
77. Kohn J, O'kelly T. An ortho-tolidine method for the detection of occult blood in faeces. J Clin Path. 1955; 8 (3): 249-51.
78. Foot C, Wiener K. Phadebas amylase test kits. Clin Chem. 1979; 25 (5): 818-22.

79. Fang R, Manohar CF, Shulse C, Brevnov M, Wong A, Petrauskene OV, et al. Real-time PCR assays for the detection of tissue and body fluid specific mRNAs. Paper presented at: Int Congr Ser. 2006.
80. Fleming RI, Harbison S. The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. Forensic Sci Int-Gen. 2010; 4 (4): 244-56.
81. Luo X, Li Z, Peng D, Wang L, Zhang L, Liang W. MicroRNA markers for forensic body fluid identification obtained from miRCURY™ LNA array. Forensic Sci Int Genet Suppl Ser. 2015; 5(2): e630-2.
82. Albani PP, Fleming R. Novel messenger RNAs for body fluid identification. Sci Justice. 2018; 58 (2): 145-52.
83. Sauer E, Reinke A-K, Courts C. Validation of forensic body fluid identification based on empirically normalized miRNA expression data. Forensic Sci Int Genet Suppl Ser. 2015; 5(7): e462-4.
84. Palmer M, Prediger E. Assessing RNA quality. Ambion Tech Notes. 2004; 11 (1): 1-6.
85. Li Z, Bai P, Peng D, Long B, Zhang L, Liang W. Influences of different RT-qPCR methods on forensic body fluid identification by microRNA. Forensic Sci Int-Gen. 2015; 5(7): e295-7.
86. Akutsu T, Motani H, Watanabe K, Iwase H, Sakurada K. Detection of bacterial 16S ribosomal RNA genes for forensic identification of vaginal fluid. Leg Med. 2012; 14 (3): 160-2.
87. Wobst J, Banemann R, Bastisch I. RNA can do better—An improved strategy for RNA-based characterization of different body fluids and skin. Forensic Sci Int Genet Suppl Ser. 2011; 3 (1): e421-2.
88. Xu J, Zhao R, Xue Y, Xiao H, Sheng Y, Zhao D, et al. RNA-seq profiling reveals differentially expressed genes as potential markers for vital reaction in skin contusion: a pilot study. Forensic Sci Res. 2018; 3 (2): 153-60.
89. Malkov VA, Serikawa KA, Balantac N, Watters J, Geiss G, Mashadi-Hossein A, et al. Multiplexed measurements of gene signatures in different analytes using the Nanostring nCounter™ Assay System. BMC Res Notes. 2009; 2 (1): 80-9.
90. Hanson EK, Ballantyne J. Rapid and inexpensive body fluid identification by RNA profiling-based multiplex High Resolution Melt (HRM) analysis. F1000Research. 2013;2(1): 281-6.

Author's Contribution

AR: Conception and design of study, critical revision of manuscript.
MJ, SN, MFA, MA, HMA, SA: Acquisition and analysis of the data, drafting of manuscript, Revising it critically for important intellectual content.
ALL AUTHORS: Approval of the final version of the manuscript to be published.