# Microbiome Biomarkers-

# Post Mortem Interval

By

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**Declaration**

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed: Maitri Solanki

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# Part One: Literature Review

## Microbiome Biomarkers – Post Mortem Interval

**ABSTRACT**

Microbiome is the catalog of microbes and their genes. And a biomarker is a substance that can be objectively measured and which can act as an indicator of pathogenic processes, biological processes or pharmacological responses to a therapeutic intervention. Also, the time between physiological death and the examination of the dead body is known as the Post mortem Interval (PMI). Determination of PMI can be a complex problem due to it being influenced by a number of intrinsic and extrinsic factors. Some of the intrinsic factors include age, sex, and pathological and physiological states of the corpse. While the extrinsic factors include temperature, humidity and insect activity. Recently, molecular changes such as protein, RNA and DNA degradation have been studied quiet widely and are seem to be producing promising results in the field of PMI estimation. More specifically, studying RNA degradation after death is considered quiet useful for precise PMI estimation. Some of the different types of RNA that aid in PMI estimation include miRNAs, circRNAs, 18S-rRNA and so on. This study focuses on the potential for estimating PMI using microbiome biomarkers as a tool rather than the tradition PMI estimation by studying various stages of decomposition.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ATP | Adenosine Triphosphate |
| DNA | Deoxyribonucleic Acid |
| circRNA | Circular RNA |
| miRNA | MicroRNA |
| PMI | Post Mortem Interval |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic acid |
| 16S-rRNA | 16S Ribosomal RNA |
| 18S-rRNA | 18S Ribosomal RNA |
| RT-qPCR | Quantitative Reverse Transcription PCR |

## 1.0 INTRODUCTION

**Microbiome:**

Microbiota, often interchangeably used in place of microbiome refers to the microbial taxa associated with human. Microbiome is the catalog of these microbes and their genes (1). “Metagenomics “, originally referred to shotgun characterization of total DNA, although now it is increasingly being applied to studies of marker genes such as the 16S rRNA gene (1).

**Biomarker:**

A biomarker as defined by the National Institute of Health Biomarkers Definitions Working Group in 1998 is a substance that can be objectively measured and which can act as an indicator of pathogenic processes, biological processes or pharmacological responses to a therapeutic intervention (2).

In other words it can also be described as a substance or a structure in the body who itself or its products can be measured (2).

**Post Mortem Interval (PMI):**

The time between physiological death and the examination of the dead body is known as the Post mortem Interval (PMI) (3). The three stages that PMI can be divided into include EPMI, PMI in the advanced stage and skeletonized remains (4). The traditional methods of PMI estimation are not considered to be so accurate during advanced stages as the corpse would have eventually extensively destroyed (4). A lot of case information would

have destroyed at this advanced stage and even the crime scene would have changed due to the delay in PMI (4).

Biological markers can now be detected more precisely with the advance in molecular biology technologies. A study by (4), have discovered potential correlation between RNA degradation in a corpse and PMI. A good method to detect RNA profiles is qPCR (4).

Determination of PMI can be a complex problem due to it being influenced by a number of intrinsic and extrinsic factors (5). Some of the intrinsic factors include age, sex, and pathological and physiological states of the corpse. While the extrinsic factors include temperature, humidity and insect activity.

In previous years, PMI estimation was done using traditional methods that considered physiological changes such as algor mortis, livor mortis, rigor mortis and supravital activity which could only provide a rough estimation of PMI and also create chances where one indication was contradicted by the other (5). Because of these drawbacks that scientists faced, recently, another method of PMI estimation has been generated that makes use of nucleic acid degradation (5).

Recently, molecular changes such as protein, RNA and DNA degradation have been studied quiet widely and are seem to be producing promising results in the field of PMI estimation (5). More specifically, studying RNA degradation after death is considered quiet useful for precise PMI estimation. The mRNA profiles are widely detected using Reverse Transcription real-time quantitative polymerase chain reaction (RT-qPCR) because it being highly sensitive, widely used and its assay's being readily available. Also, accurate target gene expression activity, endogenous reference genes are widely used as internal controls when performing data analysis (5).

Some of the different types of RNA that aid in PMI estimation include the following:

**miRNAs and circRNAs :**

miRNAs belong to the class of non-coding single stranded RNA molecules. They are encoded by endogenous genes of approximately 22 nucleotides and come under the category of satisfactory stable molecules that are minimally affected by environmental conditions, which interfere with PMI estimation (4).

CircRNAs also fall under the category of stable RNAs due to them having a stable structure that consists of a covalent bond linking 3' to 5' ends through back splicing (4). CircRNAs are a unique class of non-coding RNAs, which possess characteristics of high stability, abundance and tissue-specific expression patterns that make them valuable for the estimation of PMI in advanced stage (4).

**18S-rRNA and microRNA:**

18S-rRNA is one of the key ribosomal RNA that is a part of ribosomal protein complex (5). microRNA on the other hand one consists of 21-25 nucleotides and is a part of a class of small non-coding single stranded RNA. microRNAs are found to be present in many different species (5).

## 2.0 History of Microbes in Forensic Science

Since the late 19th century, Microbes have been used as evidence. During this period, microbiology was used as a forensic tool for determining the pathogenicity and the causes of death in humans and animals (6). A lot of this early work can be correlated with some of the well-known scientists like Louis Pasteur, Robert Koch and Joseph Lister (6). Also during this period, studies were being carried out by Edmond Locard in order to justify that microbes can also be used as trace evidence. 'Every contact leaves a trace' is one of the very well known sayings from Locard (6).

## 3.0 Soil Microbiomes as Evidence

Initially, the method used by court system for capturing microbial diversity was through the use of terminal restriction fragment-length polymorphism. RFLPs cleaves DNA by using restriction enzymes on specific sets of nucleotide bases (7). Microbiomes of the soil are a great source of evidence when estimating PMI, locating clandestine graves and linking objects, humans and locations through trace evidence (7).

## 4.0 Skin microbiomes as trace evidence

Skin microbiomes have a very unique property of being extremely individualistic (6). The microbes found on the hands of two individuals can differ more than 80 per cent. Skin microbiome serves great purpose as trace evidence due to it having properties like

unique between individuals, being transferred as clouds of microbes and remain stable overtime (6). It is also of best use if collected at the same time (same season of the year) as the surface sample. The positioning of the skin microbiome within the friction ridges provides a high chance of positive identification of the perpetrator (6).

Skin microbiomes can also serve other important purposes such as identification of a person's gender and lifestyle, whether they were located i.e. whether in an urban or rural setting and also if individuals were cohabitating (8). Moreover, skin microbiomes can also provide lots of other information about an individual such as beauty products used by them, their disease status, food consumed by them and activities they have been part of. The information gathered can be of great use in a criminal investigation (8).

## 5.0 Microbiome evidence in the criminal justice system

For microbiomes to be used as evidence in court, there is a need for it to be presented by either an investigator or a lawyer who can support the relevance and reliability of the microbial evidence (6). For microbiome evidence to be presented in court, it should be thoroughly researched and error rates should be known through quantitative machine-learning methods (6).

## 6.0 Traditional methods for estimating PMI- the physical stages of decomposition (Physiological changes)

The process of decomposition involves the degradation of soft and hard tissues of a human body in basic components through various physiochemical changes. A number of external factors such as temperature can have a significant impact on the rate of degradation (9). The time that a mammalian body takes to decompose is usually constant over all the bodies except for the effect that temperature possess on it. Temperature can vastly affect the time it takes for a body to get to skeletonization stage from fresh stage (9).

Stages of decomposition are categorized in such a way that helps investigators explain the process and estimate PMI. The process of decomposition usually commences straight after death unless the environmental conditions don't allow it to proceed (9). The two main stages of decomposition are pre and post skeletonization where the pre skeletonization stage can be subsequently divided in five sub-stages namely: fresh, bloat, active decay, advanced decay and dry.

**Fresh Stage:**

Autolysis and putrefaction are the two processes that occur simultaneously during biochemical decomposition. The initial process that can be identified during fresh stage of decomposition is Autolysis (also known as enzymatic self-digestion). Autolysis leads to loss of membrane structure in the most metabolically active cells of the body because of

the lack of energy production in the form of adenosine triphosphate (ATP) (9).
Temperature can affect the process of decomposition differently at different locations. Places with high ambient temperature accelerates autolysis, and the process is very slow at places where temperatures are cooler. At times, in regions with freezing, the process of decomposition can also be suspended due to the inactivation of the enzymes (9).

A number of processes are involved in the release of nutrient-rich fluid from the body (9). These include, the loosening of epidermis from the dermis, marbling of the skin as a result of autolysis of red blood cells and a number of post mortem blisters (bullae), which causes the cellular membrane to dissolve. This fluid that is released from the body, is a great energy source for the microbes aiding putrefaction (9).

The extent to which fresh stage extends is starting from death until the skeleton bloats at the bloating stage (10). As a result of the production of gaseous by products due to the microbial metabolic activity, the carrion gets inflated and attracts necrophagous insects towards carcass during the bloat stage (10). The next stage following bloating is the active decay where immense insect activity causes rapid decomposition (10).
The entomological activity is significantly decreased during advance decay (10).
The stages of decomposition are generally defined by the presence and activity of certain insects, which are used to calculate PMI. Hence the process of decomposition is a continumm and not discrete series of events (10).

## 7.0 Estimation of PMI in advanced stage using miRNAs and circRNAs

It is very challenging to estimate accurate PMI after about 24 hours of death i.e. in the advanced stage, because of the influence of external factors on the corpse.
The external factors include temperature being the most common, humidity and microorganism (4). However, nucleic acids like DNA and RNA are a great source for determination of accurate PMI due to them being least affected by the external factors. This is because the nucleic acids are protected inside the cell nucleus (4). In order to precisely determine PMI using RNA, it is generally preferred measuring the postmortem RNA degradation quantitatively followed by building mathematical models. The reason for this is that RNA that is present in tissues degrades with a delay in PMI (4).

RNA profiles can be detected using various methods. However, Real time quantitative polymerase chain reaction (qPCR) is one of the best ones used. a number of mRNA markers are used as endogenous control markers namely G*apdh*, Beta-actin and *Rps*18 (4). A drawback for using these as reference for quantitation of degraded RNAs is that their efficacy is reduced as they degrade during advanced stage of PMI. According to so and so, these markers are only stable during 8 days after death. A solution to this issue is to use stable and conserved RNAs such as microRNAs (miRNAs) and circular RNAs (circRNAs) as normal reference genes (4).

Based on the study done by (10), concluded that housekeeping genes have a very close relationship with estimating PMI and hence makes them potential biomarkers for PMI

estimation (10). This is so because it was observed that housekeeping genes were observed degrading with PMI extension and hence were known to have a close relationship with PMI (10). Housekeeping genes are present and stable expressed in all the cells of the body and their products play a very important role in the correct functioning of basic cell activities (10).

Certain reference genes are known to be optimal in specific tissues, for example, miR-122, miR-133a and 18S are the reference genes for heart tissues, LC-Ogdh, circ-AFF1 and miR-122 are for liver tissues and miR-133a, circ-AFF1 are for skeletal muscle tissues (4).

Furthermore, based on the study results of (12), it was found that the target biomarkers in heart and liver tissues are U6 and Rps18, while that in skeletal muscle tissues are U6 and beta-actin (12). This study also shows U6, which belongs to a small nuclear RNA family to have the highest correlation with PMI as, the U6 transcript was noticed to have been degraded alongside PMI extension (12). The advantage that U6 has is that it gets protected from external factors through the ribonucleoproteins, but this eventually vanishes with delay in death time. While Rps18, a ribosomal protein with high expression stability is observed to be degrading during late PMI and thus is considered appropriate as a biomarker in the heart and liver tissues of a corpse. When considering the skeletal muscles, Beta-actin is proven to be the right biomarker when estimating PMI (4).

## 8.0 Postmortem interval determination using 18S-rRNA and micro RNA

Research suggests that mRNA is efficient for postmortem analysis even on extreme conditions where the corpse is left at room temperature for several days (5). 18S-rRNA is found in abundance in cells of the body. It is protected from the RNA enzyme inside the ribosomal complex (5). The quantity of 18S-rRNA increases as it is released from the complex with the extension of postmortem interval and the degradation of proteins (5). Based on the experiments of (5), 18S-rRNA levels are noted to be gradually increasing during early stages, however peaks around 96 hours after death (5).

One of the stable marker's used that can be used as a n internal standard is miR-1. The MicroRNA (miR) is a highly conserved small non-coding single stranded RNA that is only 21-21 nucleotides long. miR is capable of silencing a gene by binding to its target mRNA (5). The dynamic temporal and spatial expression patterns of microRNAs can be disrupted due to developmental/physiological abnormalities. When compared with mRNA, microRNA is short in length and considered to be more stable than mRNA (5).

- **RT-qPCR in PMI estimation:**

In an attempt to determine PMI, RT-qPCR has been seen to have a major role when considering sensitivity and specificity. It is highly recommended considering factors such as temperature, age, sex, cause of death and pathological state when analyzing RNA levels from autopsy samples (5).

## 9.0 Advances in Microbial Forensics:

Microbiome forensics is the field where microbial communities can be studies in unprecedented depth due to the advances in sequencing platforms and computational pipelines (6). On the other hand, microbial forensics targets and identifies individual taxa of interest through the use of various techniques. For example, microbial forensics identifies specific strains of microbes using fine-scale variation within individual genomes, where as microbiome forensics studies the difference in community of microbes through microbial post mortem changes and trace evidence (6). The microbiome data can be used for a number of reasons namely for calculating PMI, locating clandestine graves and linking objects or spaces by using skin microbes (6). Patterns in microbial communities can be studied by using next-generation sequencing of phylogenetically and taxonomically informative gene markers such as 16S rRNA, 18S rRNA and ITS (6).

## 10.0 Metabolomics:

Alongside RNA, metabolomics is another emerging field that has great potential for determination of PMI in various forensic investigations (13). This is through identifying new biomarkers that are related to chronological changes after death. The cause of death and the changes following death in humans can be determined using metabolite profiling. On top of just the chemistry of the corpse, the metabolomics patterns of the soil under the decomposing human corpse can also be identified. Distinctive patterns like fatty acid signatures associated with PMI can also be derived using metabolic profiling approach (13).

## 11.0 Micro-RNA IN Forensics:

A lot of potential for forensic applications have been observed through promising results obtained from miRNA and small non-coding RNAs (14).

There exists 800-1000 miRNAs in the human genome out of which more than 700 miRNAs have been identified. This forms 2-3% of all protein-coding genes (14).

## 12.0 Conclusion and Further Research:

Based on the research till date, the factors affecting RNA degradation in a dead body include PMI and temperature. The effect of other minute parameters can be eliminated through controlling other experimental conditions in the research.

PMI estimation can sometimes be tricky due to many factors being involved and these factors impact in different ways. It is difficult to replicate this in a laboratory setting, as real scenario is quiet different and more difficult as many more factors are involved. A standard and well-constructed protocol should be used for obtaining quality results from RT-qPCR experiments. The experimental design should include RNA extraction, gDNA removal, target gene information, complete reaction conditions, primer design and appropriate controls for qPCR assay followed by data analysis. Further research needs to be carried out using more types of RNA in an attempt to figure out if more accurate results are obtained. The experiments should be strictly controlled and a well-structured protocol should be used.

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**Part 2: Manuscript**

# Microbiome Biomarkers – Post Mortem Interval

**Contents**

**Abstract:**

Post Mortem Interval also known as PMI refers to the time between physiological death and the examination of the body. PMI estimation can be done using communication as evidence, microbiomes, biomarkers, chemicals, and nucliec acids. Each of these methods have the capability to contribute to essential information related to an investigation. Microbes have long been used as a physical evidence in forensic science because they are ubiquitous in nature and also because they have predictable ecologies. Another form of estimating PMI is through the use of biomarkers. As identified by the national Institute of Health Biomarkers Definitions Working group in 1998, a biomarker is a substance which can be objectively measured and which can act as an indicator of pathogenic processes, biological processes or pharmacological responses to therapeutic intervention. The stages of decomposition aid to determine the cause of death, the time of death, etc. The stages of decomposition are also affected by extrinsic and intrinsic factors of the dead body. The rate of decomposition and hence the microbial succession can be influenced by environmental variables like temperate and presence of soil. PMI regression models should take into consideration environmental parameters that affect microbial succession. As a result of the advances in sequencing platforms and computational pipelines, microbial communities can be studied in great depth when concerned with microbial forensics.

**List of abbreviations**

DNA- Deoxyribonucleic Acid

circRNA- Circular RNA

miRNA- MicroRNA

PMI- Post Mortem Interval

RFLP- Restriction Fragment Length Polymorphism

RNA- Ribonucleic acid

16S-rRNA- 16S Ribosomal RNA

18S-rRNA- 18S Ribosomal RNA

RT-qPCR- Quantitative Reverse Transcription PCR

## 1. Introduction:

Post Mortem Interval also known as PMI refers to the time between physiological death and the examination of the dead body (1). PMI estimation can be done using various methods such as communications (cell phone activity, electronic or hard copy communications, visual sightings), biological evidence concerned with the human body and environment which includes rigor mortis, lividity and insect activity (1). Each of these methods have the capability to contribute to essential information related to an investigation. All of the above mentioned methods have their limitations relating to its applicability and its accuracy based on the conditions and time frames of PMI (i.e. months, weeks, days) (1). One of the methods in recent era that has proven to generate promising results for PMI estimation includes the use of degradation of nucleic acids like DNA, RNA and proteins. Research also suggests that in the future, the traditional methods for PMI estimation can be replaced by methods using degraded nucleic acids for the estimation of PMI (1).

PMI estimation in the initial hours after death is based on the rate of physical observable modifications such as algor, rigor and livor mortis. According to (1), the methods based on physical observable modifications are not considered very reliable and accurate. As a result, a valuable technique has been developed which uses RNA as a tool in forensic pathology in order to study the mechanisms of death, estimate the age of biological stains and identify the bodily fluids (2).

Microbiota is often used in place of microbiome which refers to the microbial taxa associated with human (3). Furthermore, microbiome is the catalog of microbes and their genes (3). Microbes have long been used as a physical evidence in forensic science because of them being ubiquitous in nature and also because they have predictable ecologies (3).

Another form of estimating PMI is through the use of biomarkers. As identified by the national Institute of Health Biomarkers Definitions Working group in 1998, a biomarker is a substance which can be objectively measured and which can act as an indicator of pathogenic processes, biological processes or pharmacological responses to therapeutic intervention (4). The recent advances in molecular biology technologies have made it possible for precisely detecting biological markers (4).

Some of the different types of RNA that aid in PMI estimation include miRNA, circular RNA and 18S-rRNA. miRNAs are non-coding single stranded RNA molecules (4). CircRNAs are stable RNAs that have a stable structure consisting of covalent bonds linking 3' to 5' ends through back splicing (4). While 18S-rRNA is a key ribosomal RNA which is a part of the ribosomal protein complex (5).

This dissertation focuses on how various techniques can be used to estimate the post mortem interval. It assesses the different traditional and modern day techniques by analysing their limitations, research gaps and how these techniques can be beneficial for PMI estimation. The main focus is on techniques that uses microbiomes and biomarkers for post mortem interval estimation.

## 2. Stages of Decomposition

The stages of decomposition aid to determine the cause of death, the time of death, etc. The stages of decomposition are also affected by extrinsic and intrinsic factors of the dead body. It is also known that these stages do not have a specific duration and hence is not as useful in determining the exact time of death (6). The four stages of decomposition include pallor mortis, algor mortis, rigor mortis and livor mortis (6).

**Pallor Mortis:** the initial stage after death where paleness is observed in the face and on other parts of the body. It is caused due to stop in blood flowing through capillary circulation. It occurs within 15-30 minutes of death (6).

**Algor Mortis**: After the death of an individual, they are unable to maintain the inside body temperature and hence the body itself starts to either cool or heat based on the outside temperature (6). This stage can be helpful in determining PMI by assessing the rate at which the body temperature acclimatize with the outside environment (6). There are many factors that affect PMI determination which include variations in the outside temperature, the thickness of clothing on the deaceased and the location of the corpse (6).

**Rigor Mortis:** after the death of an individual, the muscles become weak and body stiffens where by the muscles in the body contract and stay in the same position (6). The stiffening of these muscles and the body is also known as rigor mortis. It occurs after a couple of hours after death and completes after around eight hours after death. The time

that the body will stay in this position depends upon factors such as ambient temperature and the rate of decomposition of the body (6).

Liver Mortis: it is the final stage of death. The accumulation of blood in the body results in the skin appearing bluish in colour. This in other words is also known as Livor mortis (6).

Although the stages of decomposition start separately but they continue to occur simultaneously and are often overlapping in their occurrence (6).

## 3. Estimation of post mortem interval using Biochemical markers

Some of the early biochemical methods have not been taken into consideration when determining PMI because they have failed to produce reliable, precise and rapid results needed for forensic analysis (7). Recent research has however identified more sensitive and specific biochemical methods, which can be assessed statistically and can also be standardised to produce accurate results (7).

Some of the very useful information about phases like agonal period, supravital reactions, leakage from cell degradation, diffusion and decomposition of biochemical markers can be obtained from the biochemical and metabolic profiles generated from body fluids after death (7). This thus results in identifying the changing metabolic environment of the host. The identification of relevant post- mortem biomarkers can also aid in identifying potential cause of death and PMI estimation. According to (7), biomarkers in recent studies are identified using Nuclear magnetic resonance (NMR) and mass spectrometry (MS) that ultimately result in precise PMI estimations.

## 4. Biochemical markers of PMI:

There are three factors that biochemical changes can be attributed to namely, biochemical changes during early post mortem period, the agonal period of anoxia and distribution of diffusible substances into erythrocytes, plasma, interstitial fluid, tissue cells and blood (7). The various biochemical markers are as follows:

**Metabolites-**

Sodium chloride not so reliable for PMI estimation as the ante mortem serum concentrations of sodium chloride vary based on hydration status, kidney functions and illness (7).

Potassium is not so reliable biochemical marker for PMI calculations because it is very difficult and nearly impossible to calculate potassium's serum ante-mortem concentration. This is because it is immediately released from the cells after death (7).

Decomposition constitutes of enzymatic breakdown of lipids, carbohydrates and proteins (8).

In order to determine PMI and understand the decomposition process, it is must to identify the biochemical products present in the decomposition fluid and the time when it was produced (8).

Decomposition consists of complex reactions that causes the breakdown of proteins to amino acids, nucleic acids to nucleotides and carbodydrates to monosaccharides and lipid based macromolecules to fatty acids (8).

## 5. Role of microbes in PMI determination:

Microbes being ubiquitous and having predictable ecologies make them ideal as physical evidence when concerned with forensic science (3). Advancement in research has lead to the development of new microbial based tools for use in forensic science (3). This has been possible due to the boost in microbiome science and the advancement of next generation sequencing technology (3). Microbes have been proven to be very promising when it comes to the determination of PMI. Regression models made using microbiome data from post mortem samples with known PMI are used to develop the microbial clock of death (3). Microbial clock's are used in a death investigation where in microbes are profiles using DNA sequencing from samples that are collected which is then matched to a point on the clock (3). The significant tophonomic landmarks of decomposition can be organized using some arbitary stages, as decomposition being a continuous process. Cadaver decompositon can be classified into stages starting from fresh stage, bloat, active decay, advance decay, skeletonization (3).

In situations where insects are able to get to a corpse, the blow flies lay eggs that eventually develop into maggots and blowflies. The lifestyle of the oldest maggots can be used to generate a growth curve based on temperature which can then be used to estimate PMI (3). There are a number of limitations to this approach which is that this method is only useful in PMI determination within the initial two weeks of decomposition, temperature is a big influence, it is mainly preferred only if the body is present in the out environment where insect can have easy access to the corpse. This is

also a high chance of misinterpretation of maggots as what stage are they in their lifecycle (3).

**Environmental influence on PMI when using microbial models:**

The rate of decomposition and hence the microbial succession can be influenced by environmental variables like temperate and presence of soil. PMI regression models should take into consideration environmental parameters that affect microbial succession (3).

**Microbial evidence in the Justice system:**

Developing and transitioning new forensic science technologies into the justice system requires overcoming scientific, investigative, and legal hurdles. If a new technology is to be introduced into the justice, a number of procedures needs to be implemented. These include identifying need, basic research, prototype development, validation, acceptance and adoption (3).

An investigator or a lawyer who can support the relevance and reliability of the microbial evidence is required in order to use microbiomes as an evidence in court. Microbial evidence should be fully researched and its error rates should be understood through quantitative machine-learning methods for it to be presented in court.

## 6. Profiling of RNA degradation for estimation of PMI

RNA has the potential for PMI estimation, including identification of fluids, wound age estimation by studying time dependent expression of troponin 1 mRNA from skeletal muscles being one of the possible marker (1).

It is quite useful to study RNA decay as a means to estimate PMI estimation as RNA degradation or loss of RNA transcript after death are rapid and time dependent (1). RNA in the cells of an individual after death is degraded by ribonucleases that are present within the cell or from those originating from bacteria or other environmental contamination (1). Apart from the role that endogenous and exogenous ribonucleases play in in vivo RNA degradation, there are also other factors present that can influence degradation which include environmental, chemical and thermal factors as they also immediately influence RNA degradation after death (1).

## 7. Identification of Molecular Biomarkers for estimation of PMI using blood samples

The degradation of RNA in the body after death is considered to be a very useful marker for accurate PMI estimation. Based on (9) study, RNA generally gets rapidly degraded compared to DNA after death and also in vitro as a result of ubiquitous ribonuclease activity. It has been researched that RNA markers decrease in a time dependent manner while RNA levels can still be detected after 30 days of collection of sample (9).

The study by (9) also confirmed that certain RNA markers at certain temperatures degrade in a time dependent fashion through 30 days after it was first collected. Some of

them include 18SrRNA and HPRT1, B2M and GAPDH mRNAs actively degrade at 25 degrees Celsius while GAPDH mRNA degrade at 4 degree Celsius (9).

A number of different factors also affect these mRNAs which include intracellular substances like enzymes and proteins that are released from the hemolysis of RBCs in whole blood (9).

**8. Determination of PMI in the advance stage using miRNAs and circRNAs:**

As the nucleic acids like DNA and RNA are present in the cell nucleus, they are less likely to be damaged by external factors and thus have to potential to estimated more precise PMI. According to (4) RNA present in the tissues degrade with delay in PMI. It is also figured out that one of the precise methods for PMI estimation at this point in time is through the use of RNA degradation measured quantitatively and through mathematical models that potray relationship between RNA degradation and PMI (4).

One of the good methods for detecting RNA profiles is Real time quantitative polymerase chain reaction (qPCR). When considering biochemical research, some of the mRNA markers that are considered to be appropriate endogenous control markers are Gapdh, beta actin and Rps18 (4).

When considering RNAs for PMI estimation, it has been research by (4) that microRNAs and circular RNAs have proven to be more stable as reference genes when compared to other RNAs. Their research also suggests that real-time quantitative Polymerase Chain Reaction (qPCR) accurately predicts PMI in its advanced stage with low error rates and by using effective reference genes and target biomarkers (4).

Circular RNAs are highly stable and abundant with possessing tissue specific expression patterns because of which they are considered to be very valuable when estimating PMI in advanced stage (4). Also, some of the housekeeping genes are found to be very closely related to to PMI and hence have proven to be potential target biomarkers for the determination of PMI (4). Some of these target biomarkers include U6 and Rps18 in heart and liver tissues, that of skeletal muscle tissue include U6 and Beta actin (4).

Some of the limitations include the effect of temperature on RNA degradation but are controlled to a certain extent under experimental condition in research (4).

**9. Postmortem interval determination using 18S-rRNA and microRNA:**

Based on recent research, mRNA can also be used for post mortem analysis even if the corpse is left at room temperature for several days (5). 18S-rRNA is protected from RNA enzymes inside the ribosomal complex and is found in abundance in the body. When the extension of PMI and the degradation of proteins, the quantity 18S-rRNA increases and is released from the ribosomal complex. According to (5), during the early stages after death, 18S-rRNA levels gradually increase and peaks around 96 hours after death.

**Advances in Microbial Forensics:**

As a result of the advances in sequencing platforms and computational pipelines, microbial communities can be studied in great depth when concerned with microbial forensics (10). There are two different fields in forensics when concerned with microbes which include microbial forensics and microbiome forensics (10). Microbial forensics relate to the identification of specific strains of microbes. On the other hand Microbiome

forensic studies the difference in microbial communities through the microbial PM changes and trace evidence (10). There are a number of reasons where microbiome data can be used namely for calculating PMI, locating clandestine graves and using skin microbes to link objects or spaces. Next generation sequencing of phylogenetic and taxanomic informative gene markers like 16S rRNA, 18S rRNA and ITS can be used to study patterns in microbial communities (10).

## 10. Conclusion:

It has been researched that the factors affecting RNA degradation in a dead body are PMI and temperature. Other factors too have an effect on the estimation of PMI but it can be eliminated by controlling other experimental conditions in research. The traditional methods of PMI estimation that uses microorganisms and stages of decomposition are considered only to be useful when estimating PMI during the early stages after death. However as time elapses, research suggests that nucleic acids such as DNA and RNA are a good source for PMI estimation as they are protected inside the nuclear envelope and are least affected by factors such as temperature. Advances in Molecular Biological techniques have also proven to be very useful when analysing the nucleic acids.

## 11. Limitations/ Research Gaps/Further Research:

PMI estimation that takes into consideration rigor mortis and the decomposition process in the body has many factors that influence it such as the outside and inside environmental conditions.

Detection of post mortem changes in protein, RNA and DNA markers has not yet been systematically studied.

Studies should incorporate environment variables such as oxygen, humidity, precipitation and presence of insects when estimating PMI using regression models.

In future studies, the kinds and numbers of biomarkers and tissues to estimate PMI in its advanced stage should be increased.

Further research is needed into how succession of microbes in different environments like terrestrial, aquatic and indoor affect mammalian decomposition.

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