Metagenomics Analyses: A Qualitative Assessment Tool for Applications in Forensic Sciences

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Abstract

Forensic science deals with the scientific investigation of evidence collected from crime scenes. Since decades, forensic scientists have solved complex and sophisticated crimes using biological evidence. The forensic analyses include the biochemical profiling, physicochemical profiling and molecular analysis of the evidence. The biochemical profiling involves the analysis of the lipid-profile, the presence of toxins in the visceral fluids and enzyme activity analyses. However, it has come to light that detailed molecular evidence, besides the conventional DNA-evidence, is required for the accurate implication of an individual in a crime. Pre-meditated crimes with cadavers found in outbound locations are especially challenging. Many times, the biological samples do not provide enough DNA to carry out the molecular analysis. Metagenomics has paved a way to explore the microbiome of the biological evidence from which the DNA-evidence cannot be extracted. Using high-throughput sequencing methods followed by sequence analysis, the environmental samples of evidence can give valuable insights into the detailed molecular patterns. These patterns can make the identification or comparison of subjects easier. The chapter discusses the genomic analysis, proteome analysis and the metagenomic approaches for investigating a crime scene.

Keywords

DNA profiling · Human microbiota · Metagenomics · Microbial forensics · Molecular markers
5.1 Introduction

Forensic science involves the utilization of the laws, principles and tools of science and technology to examine the various physical, chemical and biological evidence around a crime-scene for an amicable solution in the court of law (O’Brien et al. 2015). Nowadays, in cases such as a bioterrorism event, an oil spill or chemical discharge, or a food-borne infectious disease, microbes serve as some of the biological evidence. Analysis of the microbes in such scenarios may be termed as “Microbial Forensics” (Teaf et al. 2018).

Prospecting of the biological evidence at crime scenes should be prioritized, validated and configured using microbial forensics. It plays a significant role in configuring, sample handling and solving the crimes using biological evidence (Budowle et al. 2007). Microbial forensics is a discipline of forensic science that utilizes microbiology to aid in the investigation of biological crimes. It primarily includes the identification and analysis of different microorganisms and their toxins as well as the storage, preparation and mode of delivery of these microbial toxins and pathogens (Pattnaik and Jana 2005).

5.2 Microbes as Weapons

Microbes are potential weapons and not the newly discovered ones, even though less sophisticated. An unfortunate aspect of a biological weapon, when compared to a nuclear weapon, is that it requires much less capital and other investments. Microbes can be cultivated in a small establishment, with meagre investments, and it does not need any elaborate or sophisticated instruments (Thavaselvam and Vijayaraghavan 2010). The need for highly-skilled human resources is also not necessary. Microbes and pathogens can procreate and multiple from a single cell to an uncountable amount if suitable conditions are available. The most common method of using these biological weapons is releasing the virulent pathogens and microbes into the environment that eventually affects the whole community (Riedel 2004).

Any microorganism or microbiological agent can be used as a potential weapon. The factors determining its effectiveness include the ecological stability of the pathogens and the pathogenicity (Cannons et al. 2007). With the advancements in genetic engineering and genetics, common bacteria and organisms can be engineered to produce highly toxic products by inserting ectopically sourced genes. Engineering with transgenes can render these microbes extraordinarily effective and environmentally stable. Biological warfare could be deadly as it uses transferrable and contagious lethal pathogens or toxins to attack the target population. The self-sustainability of these biological attacks can cause severe damage at all levels (van Aken and Hammond 2003).
5.3 Microbes as Environmental Trace Evidence

Different microbes in the environment may serve as trace evidence of a crime committed in that environment. Soil microbiota indicates the evaluation of the degree of decomposition of an excavated/exhumed cadaver and identification of clandestine graves (Hampton-Marcell et al. 2017). Analysis of the microbiome in a water source can assist in determining the extent of pollution of the source or its suitability for human consumption (Poussin et al. 2018). Analysis of the microbes, such as diatoms, is commonly used in cases where a corpse is found in water. This analysis establishes if the drowning was antemortem or postmortem, and if drowning is the cause of death, whether or not the person was killed in the body of water, they were found in (Armstrong and Erskine 2018).

The microbial ecology is dependent on the human microbiome, and the constant interaction between humans and the environment leave specific “microbial signatures” on the human or their accessories (cellphone and shoes). These microbial signatures serve as a means of tracing the person’s movements and previous locations (Ursell et al. 2012; Lloyd-Price et al. 2016).

5.3.1 Microbes in Humans

The study of the human microbiome is a dynamic research area. It can yield significant insights into different, forensically relevant aspects such as the identity, geolocation, health and diet of an individual and also to determine the postmortem interval (PMI) and to track the source of infection in case of bio-crimes (Pechal et al. 2018).

Humans harbor microorganisms as part of their physiological development. These microflora collectively constitute the human microbiome. The normal microbiota can be analyzed to identify a person as the collection of these microflora vary from person to person and hence serve as a forensic signature (Thursby and Juge 2017). These microflora are routinely shed, deposited and exchanged continuously, following the principle of exchange given by Locard. Furthermore, since there is a diversity of microbes that are native to different organs of the human body, supporting information like the origin of biological evidence may be determined, that could be used to determine the extent of involvement of humans in crimes (Meadow et al. 2015). An illustration of the expanded horizons of forensic testing using human microbiota is given in Fig. 5.1.

5.3.2 Paleomicrobiology

It refers to the study of ancient microbes and their DNA. Paleo-microbiologists study the epidemiology of ancient infectious diseases and trace the migration patterns of diseases in the past. They use petrified feces (called coprolites) to analyze the gut microbiomes for understanding the dietary aspects of our ancestors (Warinner et al.
2015). Chas B. Lipman isolated microbes from coal that dated back to millions of years.

Specimens of human origin, have been used by forensic paleo-microbiologists to investigate diseases that have afflicted humankind in the past, including parasitic, viral and bacterial infections (Rivera-Perez et al. 2016).

5.4 Forensic Analysis of Microbes

The Federal Bureau of Investigation (FBI) of the United States of America (USA) has launched the scientific working group on microbial genetics and forensics (SWGMGF), which lays down the infrastructure for microbial forensics (Budowle et al. 2007). The SWGMGF aims to impart knowledge and guidance of the criteria for optimum extraction of information from the available physical evidence. The information can include the identity of the microbial toxins, the convict/victim(s) and the tools used in the execution of a criminal act. The development of a microbial forensics program by SWGMGF enables validation of different methods used in forensic analyses. Apart from the development and validation of methods, the SWGMGF focusses on the quality assurance (QA) as well. QA includes the development of technical proficiencies of the personnel, record-keeping and standard-operating procedures.

DNA profiling is a powerful tool for the identification and elimination of the origin of biological evidence. It may be combined with other effective methods, such as analytical chemistry, pattern matching techniques and microscopy, to give a more accurate characterization of the microbial evidence (Magalhães et al. 2015). A review of the DNA profiling markers has been included later in the chapter.
The isolated microorganisms are sequenced through whole-genome sequencing (WGS). The metagenomes of different niches in the ecosystem are also sequenced and analyzed. The sequencing data then allows the development of the microbial genome databases. These databases enable comparative genomic analysis for forensic analysis of the microbes (Garza and Dutilh 2015). Advanced techniques include the MALDI-TOF-MS method, protein microarrays and DNA, lipid profiling and DNA, expression arrays, SNP method, multiple-locus variable-number tandem repeat analysis (MLVA), confirmative sequencing of PCR product and proteome analyses (analysis of the entire protein profile of a microorganism or cell) (Pattnaik and Jana 2005). These application of these methods in forensic investigation is illustrated in Fig. 5.2. Nucleotide sequencing, along with the comparative evaluation of sequence polymorphism, can determine variations in signature sequences (Pareek et al. 2011).

5.4.1 Human STR

STR stands for Short Tandem Repeats and are also known as microsatellites. These are specific sequences of repetitive nucleotides found in human DNA. The STRs help in the DNA analysis and genetic characterization of individuals based on the differences in the STR patterns (Fan and Chu 2007). DNA analysis has become the go-to identification method for forensic cases where DNA extraction is possible. The human genome has several variations among individuals despite being very similar to each other (Butler 2015). The variations present in the non-coding areas of the human DNA consist of several repeating patterns of nucleotides; one such
variation is found in the form of STRs. The STRs consist of one to six nucleotide base pairs repeated several times, varying in different individuals (Willems et al. 2014). The unique number of repeats found in STRs makes an allele. STRs are now a popular method for DNA analysis and identification. FBI uses 13 STR loci in the CODIS (Combined DNA Index System) program. The CODIS program links the central, state and regional forensic science laboratories, which enables these laboratories to search for DNA variations as evidence beyond the state boundaries. STR technology is widely used in paternity tests, other parentage and kinship tests, crime scene investigation and individual identification (Roewer 2013). The STRs can be identified and extracted from a DNA sample after the STR locations are identified the STRs are amplified using PCR and then differentiated according to their size. The STRs are then analyzed and compared with the standard STRs found from the standard sample in forensic cases (Schneider 2012). If the STRs found in a suspected sample matches with that found in the standard sample, then the identification is positive and otherwise not. STR comparison and matching can also be made with the help of the several STR databases present. STRs are very prone to mutation and approximately make up almost 3% of the human genome (Kayser and De Knijff 2011). The STRs are also prone to polymorphisms. Several developmental and neurological diseases have been associated with STR expansions. STRs also carry out several important functions in humans like the gene expression regulation, DNA repair and duplication, chromatin organization and much more (Press et al. 2014).

5.4.2 DNA and Protein Microarray

Arrays or microarrays are biochips which contain a large number of biological molecules (oligonucleotides for DNA, proteases for proteins, among others) arranged in an array on glass or any other appropriate solid surface. These help to segregate individual molecules from one another and study each molecule independently (Jonczyk et al. 2016).

Microarrays provide information about genome contents with sensitivity, specificity, redundancy, reproducibility and efficiency as required for microbial analysis. Since the 1990s, they have been used for the study of cell biology. It was later used to detect infectious microbial agents (viruses and bacteria) (Miller and Tang, 2009).

A DNA microarray analysis usually follows the steps as under:

- Capture probes are designed from the database of direct sequencing genes of microbes.
- These capture probes are immobilized on the chip (in rRNA analysis, they are usually designed to match the 16S rRNA gene). A single microarray chip can contain up to 400,000 probes.
- Multiplex PCR is carried out of the DNA isolated (labelled with fluorescent dye) from the sample. In some microarrays, PCR is not required. The nucleic acid isolated from the sample is used directly.
• The amplicon (or isolate) is hybridized on to the chip.
• The pattern of hybridization is analyzed using an appropriate imaging mass spectrometry (as per the fluorophore label used for hybridization) and then compared to identify the best match.

Probe lengths may vary from 20–100 nucleotides. Short probes have forensic application and are used in case discrimination of sample using SNP or other short genetic sequences that are unique. Long probes enable detection of related targets (Ziętkiewicz et al. 2012).

Microarrays may be used to determine and measure the host’s response to pathogens which further helps to differentiate between strains of a microorganism. The advantage of this technique is that it is even possible to detect species and serotypes that are not specifically represented by the chip; they exhibit unique hybridization signatures (Rasooly and Herold 2008). On the other hand, interference caused by host nucleic acid is a serious setback. While novel sequence may be limitedly detected, its characterization is difficult or impossible, and information on the nature of the variation in the sequence is inadequate (Zhang and Appella 2010).

This technique also lacks provision of the particulars about the genomic context of a feature that has been detected, i.e., whether the gene is encoded in the plasmid or chromosomally or if it originates from horizontal gene transfer and other information about its evolutionary history (Juhas et al. 2009). Pan microbial arrays, like the Greene Chip, were used to detect Plasmodium falciparum, as the cause, during the Marburg virus outbreak. In the SARS outbreak in 2003, VitroChip microarray was helpful for detection (Palacios et al. 2007). Protein arrays work in the similar principle as DNA arrays except that probes are of proteomes of all known strains of a pathogen. The proteomic analysis is especially useful in the detection of engineered strains. The downside is that they are at risk of reduced sensitivity due to changes in the conformation that occur at the time of attachment to the chips (Hall et al. 2007).

5.4.3 DNA and Lipid Profiling

Lipids are majorly present in the membranes of the microorganisms, and some of these are distinctive to a particular microorganism. In environmental microbial studies, the phospholipid ester-linked fatty acids (PLFA) in bacterial membranes are utilized to study bacterial communities (Quideau et al. 2016).

The whole-cell fatty acid analysis is an important tool to characterize a species. It is especially used in the study of the species in genus Bacillus due to their extensive use as a bioweapon as a virtue of its pathogenic potential. Fatty acids may be analyzed by FAME (Fatty Acid Methyl Ester) profiling method, which involves the chemical extraction method (Sreenivasulu et al. 2017). In-situ extraction may be done using super-critical fluid for extracting the fatty acids from whole cells or by using pyrolysis in a microreactor, which is then analyzed by FAME. In in-situ extraction, the fatty acids are hydrolyzed and derivatized in a single step, thus saving
The extracts can be analyzed using different instruments like GC, MS, GC-MS, and HPLC (Gharaibeh and Voorhees 1996).

Lipid biomarkers can be in the form of presence or absence of single or multiple, structurally unique fatty acids. Since they are characteristic to particular genus/species, composite lipid profiles serve as a forensic signature (Abe et al. 2017).

Lipid profiles depend on:

- Type of organism
- Growth conditions
  - Environmental conditions
  - Nutrients medium characteristics

The lipid profile peaks due to growth conditions may be nullified by analyzing the total profile for a lipid specific to an organism or the variation in the relative abundance of the different biomarkers. Hence these methods can also be used for profiling a mixed sample (França et al. 2018).

5.4.3.1 SNP Analysis

The DNA of any organism consists of specific locations which provide the different sequences to various organisms within the same species. These specific locations are known as Single nucleotide polymorphism (SNP). These are the differences in human DNA which are the most common types. There are approximately three million SNPs. Most of the SNPs are considered to be biologically silent as they do not affect the inherited traits or gene function (Vignal et al. 2002). Certain SNPs may affect the gene expression in diseases or might be present in the gene, which affects the function of the protein. SNPs provide a significant ability to understand and provide treatment for human diseases and also provide a genetic marker that helps in identifying and characterizing any species specifically for applications in forensics (Alwi 2005). The stability, frequency and even distribution of the SNPs in the genome provide them with the particular value as genetic markers. An SNP map with high density with the identification of SNP positions on the genome can be used for genetic characterization and speciation of species (Kumar et al. 2012). SNPs serve as genetic markers for genome studies and experiments on fine-scale genetic mapping (Clifford et al. 2004).

5.4.3.2 Multi-Locus VNTR Analysis (MLVA)

In MLVA, multiple target VNTR loci are identified and typed and then compared with a library to identify the species. This analysis, used for microbial species, is comparable to STR typing used for human identification (Pourcel et al. 2011). VNTRs are highly mutable and hence highly discerning, which helps in differentiating between even closely related isolates. Hence when multiple VNTR loci are considered, the discriminating power is greatly intensified (Octavia and Lan 2009).
The process of MLVA may be summarized as follows:

- Isolation of DNA from the target.
- Designing of STR sequence-specific primer sets, as many as required for accurate identification.
- PCR amplification of the target STRs. Size of the PCR products may be determined based on the library of sequenced target organisms.
- Isolation of PCR products by electrophoresis (gel or capillary)
- Comparison of electrogram of PCR products for identification of target species.

The main drawback of MLVA typing for microbial species is the astounding number of species and sub-types which require an equally intensive library of standards for comparison. It would also require a larger number of multi-VNTR, which makes it highly difficult to type all microbial species in this manner (Nadon et al. 2013).

MLVA requires developing specific primers, respective to the identified STR loci and usually requires a large number of primers sets for a single species. (8–25 for *B. anthracis*, 19 for *Mycobacterium tuberculosis*, 25 for *Y. pestis*) (Guinard et al. 2017). In the case of an epidemic outbreak, where time is of the essence in identification, the presence of subtypes of species may further complicate the process, making MLVA typing unfeasible (Salaün et al. 2006).

Markers used for MLVA are subject to faster mutation than those used for SNP analysis, making it hard to exclude mismatches completely. This hindrance is further complicated by the fact that the reproducibility of MLVA results between tests done at different labs is a varying measure and each new strain has to be compared with all available results to determine the result (Zaluga et al. 2013).

MLVA can be used in cases of epidemics caused by microbes to determine whether it is naturally caused if it is a case of bioterrorism (anthrax and plague). It was most popularly used in the 2001 anthrax attack in the US. Keim sub typed *Bacillus anthracis* (used in the 2001 anthrax attack in the US). It linked their presence in clinical samples to samples collected from the patients’ food and environment, thereby helping identify the sources of the exposure of the patient to the bacteria (Le Flèche et al. 2001). MLVA used 15 VNTRs to discern 221 genotypes of *B. anthracis* and confirmed the strain that was used as the Ames strain and hence helped dissociate unrelated cases of anthrax. The identification of the strain was also crucial in determining the outbreak as a deliberate attack, as opposed to a natural outbreak, as the Ames strain is a rare strain which is scarcely found in nature (Hoffmaster et al. 2002).

### 5.4.4 Proteome Analysis

The proteome is the term that usually describes the protein complement to the genome obtained from any organism. Proteomics is defined as the cluster of measurement techniques and methods used for protein analysis (Chandramouli and Qian 2009). Microbial proteomics associates a variety of laboratory methodologies that
are based on the following scientific disciplines: biochemistry, analytical chemistry, microbiology and computational sciences. Thus, it is a multidisciplinary scientific field which requires a firm and robust understanding of the limitations, uncertainties and the abilities of the various disciplines (Graves and Haystead 2002). Proteomics identifies the peptides and proteins in a microbial isolate and compares it to other isolates or database of the protein sequence. The aim of the field is sample matching or identification related to organism state or the culture conditions. The various methods utilized for the analysis are gel electrophoresis, mass spectrometry combined with enzymatic digestion and peptide sequencing (Graham et al. 2007).

Proteins provide the expression of genotype, and also the protein complements encoded by any organism provides all the characteristics associated with it. They usually consist of linear chains of 20 different amino acids combined into a particular three-dimensional conformation. Peptides are the smaller chains of amino acids with usually <50 amino acids (Nussinov et al. 2019).

Certain proteins carry out the functions of the bacterial cell, which are division, growth, response to environmental conditions and energy utilization. For specific cellular functions, the proteins involved are produced constitutively. Thus, the identity and sequence of proteins might provide a basis of comparison and organism identification (Merkley et al. 2019).

Mostly microorganisms provide a dynamic response to environmental conditions by changing expressions of various genes. Thus, the proteins expressed by an organism could provide with the details about growth and environment, which provides information for application in forensics (Oonk et al. 2018). However, the challenge is to extract information on what parameters have been used for organism culture based on the given protein profile. A specific protein may have a role in more than one cellular process, and therefore, the expression may be similar to more than one environmental influence (Gräslund et al. 2008). Also, profiles of protein expression can provide a means to differentiate between samples of a single organism cultured under various conditions. The various factors which have been investigated to study their effect on the expression of factors of protein virulence are pH, ionic content and temperature, among others. This information, along with other forensic information, can provide knowledge about the growth history of a collected sample and for comparison purpose (Pérez-Llarena and Bou 2016).

Gel electrophoresis is a separation tool with a low resolution that provides an indication of the size and range of proteins in a collected sample (Zhu et al. 2012). However, it determines only an approximate molecular weight of proteins and is, therefore, challenging to determine the identity of a protein. This problem can be solved by using Mass spectrometry which is a high-resolution analysis tool (Gulcicek et al. 2005).

A mass spectrometer comprises of an inlet system for introducing the sample into the instrument, an ionization source for transferring the analytes as ions into the gas phase and a mass analyzer for detecting the ionized molecules based on the principle of mass-to-charge ratio (m/z) (Rubakhin and Sweedler 2010). In biological mass spectrometry, there are two mechanisms for primary ionization. These are electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI). Both the techniques are integral and produce the entire molecular ions
of biological origin. ESI can provide multiple charges to a molecule and is agreeable to on-line methods of chemical separation. In contrast, the latter provides single charged ions and needs off-line methods of chemical separation (Banerjee and Mazumdar 2012). Also, there are various types of mass analyzers which can be combined with the front-end ionization methods which vary in sensitivity, mass accuracy, resolution and other parameters. In addition, there are mass spectrometers which can carry out tandem experiments for generating more information such as the sequence of the peptides (Han et al. 2008). In these experiments, the initial step is to obtain a spectrum of the entire molecular ions in a given sample. This is followed by various other experiments in which the entire molecular ion is secluded from other components and exposed to molecular fragmentation. This breaks the secluded, entire molecular ion into small pieces which can give a lot of information about the composition of the initial ion present (Liu et al. 2007). A tandem mass spectrometry experiment has the ability to break an entire protein ion into small pieces which indicate the sequence of amino acids that comprise the initial peptide. The masses of proteolytic peptides and related tandem mass spectra can be compared with sequence database already predicted for identification of peptides (DiMaggio and Floudas 2007).

The two approaches for using mass spectrometry for proteomics analysis is usually referred to as top-down and bottom-up proteomics. The goal of both approaches is to identify the proteins in the sample. The latter involves enzymatic digestion of the protein sample such as bacterial isolate to break the proteins into small peptides before mass spectrometric analysis (Zhang et al. 2013). A small portion of the digested sample containing peptide is introduced into a separation apparatus to separate the peptides before mass spectrometric analysis. The individual peptides are identified in the mass spectrometer and then exposed to fragmentation which identifies the sequence of amino acids within each peptide (Gundry et al. 2009). The advantage of the latter is that the small peptide pieces are identified more easily by mass spectrometry with higher specificity and sensitivity. However, complete identification of the digested pieces of the original protein is rare. Thus, important information regarding protein modification (oxidation or phosphorylation) can go undetected and be lost (Angel et al. 2012).

On the other hand, top-down proteomics comprises of performing mass spectrometric analysis on an entire protein followed by tandem mass spectrometric experiments which utilize the process of secondary fragmentation to provide the information related to fragmentation or sequence of the oligopeptide (Lyon et al. 2018). The advantage of this process is the ability to detect proteins of un-sequenced organisms with huge homology to sequenced homology and for determining protein modifications via mass shifts on the intact protein. At the same time, separation of protein can be more challenging than the separation of the peptide. It would be less efficient for large molecules of protein in the gas phase and less sensitive, which would result in less peptide sequence information. Both the approaches have value in applications of bio forensics (Kim et al. 2016).

Hyphenated techniques are available to detect and measure variations in the molecules or proteins of the different strains which aid in pinpointing of its origin.
and routes of transmission (Patel et al. 2010). These include MALDI-TOF-MS, GC-MS and LC-MS-MS.

### 5.4.5 MALDI-TOF-MS

It is the abbreviation for matrix-assisted laser disruption ionization-time of flight-mass spectrophotometry. The important uses of this method are rapid microbial identification, direct identification of biomarkers from samples and rapid subtyping. The instrument has a high speed of analysis. The mass spectrophotometer consists of an ionization source, a detector kept under high vacuum and an analyzer. An electrical signal is generated by the ions which are proportional to the number of ions in the detector and a data system documents these signals. It transforms them into a mass spectrum (Clark et al. 2013). The preparation of the sample is comparatively simple. Crude or purified sample is co-crystallized with a high amount of organic matrix onto a target plate which is placed in the mass spectrophotometer. A UV laser is used to produce the ions in most of the microbiological applications (Van Baar 2000). This method could be used directly to cellular suspensions or crude cellular fractions to provide profiles of chemotaxonomic signature. It is also used for the analysis of RNA and DNA, bacteria, bacterial proteomics, rapid characterization of the bacteria at the genus, species and strain level and detection of unknown proteins and their characterization. It can be used for proper identification of light differences between related strains (Singhal et al. 2015).

### 5.4.6 GC-MS

GC-MS stands for gas chromatography-mass spectrometry. GC-MS is a hyphenated instrumental technique that combines gas chromatography and mass spectrometry. The GC-MS is essentially used to identify different components of a mixture, separate and quantify them. GC-MS is an ideal technique for the identification of elements with low molecular weight. The thermal stability and volatility of a substance are crucial for it to be compatible for analysis by GC-MS. Sample preparation for the technique is unique as materials that can affect the quality of the results may need to be removed (Brattoli et al. 2013). Solvent extraction and different wet chemical methods of sample preparation are also employed. The whole process starts with the gas chromatograph. First, the sample is injected through the inlet, and it gets volatilized. The volatilized sample is effectively separated into its components in the chromatographic column. The carrier gas is the mobile phase and is used to help the volatilized sample to reach the column. The capillary column contains the station phase. The interaction of the sample with the mobile phase and stationary phase results in elution of the sample components at different retention times. Then comes the mass spectrometer, as soon as the sample components leave the column, it gets ionized by the mass spectrometer (Koek et al. 2011). The mass spectrometer uses either a chemical ionization source or an electron ionization.
source. These different methods have different advantages and help in identifying the molecular weight of components, molecular fingerprints and structural details of the components (Banerjee and Mazumdar 2012). The mass spectrometer has a mass analyzer that separates the molecules that pass through it depending on various mass related properties; the properties analyzed depends on the analyzer used. Common mass analyzers include quadrupole or ion traps. They separate the ions according to the differences in their mass to charge ratios (Clarke 2017). The last step in this technique is the ion detection and analysis, the ions enter a detector, and several peaks are observed. The outcome from the detector is amplified for signal amplification. The signals are stored in the computer and converted to visual displays. The results can be analyzed using various computer libraries and databases and the different components identified (Beale et al. 2018).

5.4.7 LCMS-MS

LC-MS-MS stands for liquid chromatography-tandem mass spectrometry. This powerful analytical tool combines the separating capacity of liquid chromatography with the sensitive analyzing capabilities of tandem mass spectrometry (Pitt 2009). The LC-MS-MS is different from the LC-MS in many significant ways like heightened sensitivity and specificity. Triple quadrupoles are used in the LC-MS-MS system. First, the liquid chromatography is present; separation of components occurs through the different interaction of the interest samples with the customized mobile phase and stationary phase present (Grebe and Singh 2011). The sample is pumped through the stationary phase using a high pressure liquid mobile phase. After the elution of different components at different retention times due to the different chemical interactions present is directed to the mass spectrometer present. The LC-MS-MS system has a mass spectrometer with an ionization source that nebulizes, de-solvates and ionizes the effluents of the LC (Beccaria and Cabooter 2020). Charged particles are created. Electromagnetic fields help these charged particles to move through a series of mass analyzers under high vacuum. The mass analyzers used are generally quadrupoles. A parent ion or precursor ion with a specific mass to charge ratio is made to pass through the first quadrupole, excluding other particles. The parent ion is converted into daughter ions in the collision cell through fragmentation with an inert gas. Specific daughter ions are targeted using the third quadrupole and those quantified using an electron multiplier. The MS2 present, the fragmentation of parent ions to daughter ions is very specific to the structure of the compound and thus provides heightened selectivity (Girolamo et al. 2013). The MS-MS is 100× times sensitive than MS. The MS-MS targets the interest compound and breaks it down into smaller immediate compounds and filters these products. The filtered products are then identified and detected. This provides for high sensitivity and precision of the LC-MS-MS method. The fragmentation patterns provide more structural information about the component of interest (Sherwood et al. 2009).
The detection protocol should be rapid, effective and specific. High sensitivity in detection methods is required. A molecular diagnosis is the key detection method, but the ambiguity of the microbes used and the partial DNA or unknown DNA of these microbes remains a major setback (Rajapaksha et al. 2019). Prioritizing and identifying biological threats are the key goals of microbial forensics. This can be achieved using several strategies that include creating databases based on identified vulnerable population to provide information, identifying populations that may be vulnerable, developing identification protocols for biological threats using protein signatures, genetic signatures and so on (Casadevall and Relman 2010). The validity of the results and procedures must be constantly updated based present literature. The microbe used as the biological weapon may be unknown; it may not be a human pathogen but maybe a plant pathogen (Pattnaik and Jana 2005). This can cause severe economic damage. Here comes the importance of creating and authenticating a database that could serve as a guide for all the identification procedures and final results. The databases could use inputs from forensics, pure science, genomics and microbiology (Metcalf et al. 2017).

5.5 Role of Metagenomics in Forensic Identification

With the advent of methods in molecular markers as discussed above, genotyping methods and sequencing techniques, the role of metagenomic approaches is receiving much attention. A scene of the crime is full of evidence as there is no perfect crime. The evidence of importance from the metagenomic standpoint includes soil, hair, semen, blood, skin cells and many more. Soil is considered to be ubiquitous due to its presence in forensic evidence. A forensic investigator may find the soil samples in the vehicle tires, shoes, under nails, the skin as well as the discarded murder weapons (Fitzpatrick 2009). Traditionally, the analysis performed on the soil evidence includes the physicochemical analysis, basic microbiological analysis and microscopic analysis. The physicochemical analysis includes analysis of soil texture, color and estimation of the elements present in the soil sample. It is well established that such analysis, although, provides basic analysis, fails to provide an in-depth understanding of the comparison between soils (Moreno et al. 2006). Since the ultimate goal is to match the soil samples, the said matching must be done at the molecular level in detail, so that the evidence is not challenged. On the other hand, the microbiological analysis brings us close to an accurate comparison of the soil samples. However, it is limited by the fact that the number of culturable microbial species in the soil sample is only around 10%. Therefore, there is a need for bringing metagenomic analysis to cater to the need for complete microbial diversity analysis. Metagenomics is the study of the genetic composition of all microbial species in an environmental sample. These samples may include the soil, water, sediments, among others. Since soil contains a rich population of the microorganisms, the sheer diversity of the microbes makes the comparison of soil samples difficult. The method of choice used for elucidation of the microbial diversity of a soil sample is highlighted in Fig. 5.3. The basic idea is to use the metagenomic sequencing
approach for obtaining the reads from the environmental samples. The reads are then annotated using online tools such as MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) (Khodakova et al. 2014).

Similar to the soil, another potential evidence at the crime scene is the hair samples. Both scalp hair and pubic hair are acceptable evidence in the court of law. However, the comparison is usually made using the microscopic examination and hair-pigmentation (Houck 2005). Some success in fruitful utilization of hair as biological evidence is due to the molecular analysis. Techniques such as STR profiling can prospect the genome for unique gene signatures for identifying a person of interest (Linch et al. 1998). For the hair sample, the molecular marker analysis is possible only when the hair follicle is intact for extracting DNA. Lack of sufficient intact chromosomal DNA is a persistent challenge for most hair-based evidence. The lacuna is overcome using the metagenomic approach, in which culture-independent analysis of the hair-microbiota is performed. In one such study, the subjects collected the scalp and pubic hair samples using the prescribed collection kits and tools. The forensic team cut the hair samples into short fragments and extracted the total metagenomic DNA from the hair sample. The method followed in here was a direct DNA extraction method. The team then amplified the 16S rRNA gene using specific primers containing a sequencing adapter and multiplex identifier (MID) tag sequence. Unique MID tag sequences were used for the scalp and pubic hair from males and females. The amplicons were ~350 base pairs long and sequenced. The sequence reads that were obtained in this study were pre-processed using the QIIME (Quantitative Insights into Microbial Ecology). The QIIME output was loaded on the USEARCH61 for eliminating the overlapping sequences and assembling the operational taxonomic units (OTUs). The clustering analysis of the OTUs was done using the principal coordinate analysis plot (PCoA) method. Ultimately, the OTUs of the cohabiting subjects were compared with the non-cohabiting subjects. The research
found that pubic hair shows less influence from the environment and are more niche-specific. This is because the pubic hair showed more stable OTUs as compared to scalp hair. The scalp hair contained more OTUs of the environmental microbial species. Therefore, the metagenomic analysis of hair is a substantial approach to unravel the hidden microbiota as evidence (Tridico et al. 2014).

High-throughput metagenomic sequencing can also help the forensic researchers in analyzing the microbiota of a cadaver. Post-mortem, this microbiota increases exponentially, leading to foul smell due to decomposition of the tissues. Traditionally, the minimum post-mortem interval (PMI_{min}) is an entomological parameter, which implies the time passed since the death of the individual. However, the microbiota in the cadaver remains elusive except in the cases of deaths caused due to unknown infections. Therefore, the term “necrobiome” has come into existence, which refers to the microbiota in a cadaver (Benbow et al. 2013). For understanding the microbial populations in the cadaver, the high-throughput techniques like pyrosequencing have been used in a controlled experimental setting using pig cadavers. In the research, the pig carcass swabs were collected from various sites including buccal cavity, tongue and skin. The metagenomic DNA was extracted from the swabs and amplified using specific tagged 16S rRNA primers. The amplicons were cloned to prepare a metagenomic library. The pooled clones from the library were sequenced, and the sequencing reads were processed. In the processing, the short reads, non-ribosomal DNA sequences and the primer sequences were eliminated using the black box chimera check (B2C2) program. The sequences after processing were aligned using the infernal aligner tool of the Ribosomal Database Project (RDP) to generate the final sequences. From the complete sequence data, the diversity was correlated with the time of decomposition after death. Using the correlation data, the relationship between the species-richness and the time showed the progression of decomposition (Pechal et al. 2014).

5.6 Conclusion

Microorganisms are at the forefront of forensic investigation as biological evidence. Especially the human microbiota provides a reliable signature when analyzed from exhumed cadavers or a water-borne corpse. The analysis of the latter is especially useful in drowning cases. The microorganisms in the fossilized remains provide vital information during characterization of such fossils. The forensic analysis of microorganisms is carried out using molecular markers through DNA profiling. High throughput DNA sequencing can help in the identification of microbial diversity at the level of single nucleotides. Various genotyping markers such as SNPs, MLVs and STRs (minisatellites) provide detailed molecular analysis of genetic variations among different forensic samples. Apart from the DNA based markers, the biochemical profiling for lipids and the proteome profile also enable a targeted analysis of the evidence. However, for DNA analysis to be possible, the biological evidence should contain sufficient intact nuclear material. For trace evidence such as hair and soil, DNA extraction is a challenge. Therefore, the metagenomic analysis of
the microbial species associated with such samples provides the molecular signature the forensic experts look for. Metagenomic analysis of the soil and hair samples follows many similar strategies. However, the quality and quantity of DNA analysis involved in these samples are overwhelming. Metagenomic analysis of the soil and hair samples require correlation with the already existing microscopic, physico-chemical and biochemical analysis. There is also scope for standardization of forensic procedures for developing standalone methods of forensic analysis. Overall, metagenomic analysis is highly recommendable for inclusion into mainstream forensic analyses.

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