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EMERGING APPLICATIONS OF MICROBIAL FORENSICS IN CRIMINAL INVESTIGATIONS: A REVIEW

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Abstract— **Microbial forensics has greatly advanced due to recent developments in genetic data creation, especially through massive parallel sequencing (MPS), opening new investigative opportunities for criminal cases. Microbial forensics was first applied in environments related to crime, bioterrorism, and epidemiology, but it now has a wider application. Using microorganisms as evidence in criminal cases, determining the causes of death (e.g., drownings and hospital-acquired infections), aiding in human identification through skin, hair, and bodily fluid microbiomes, offering geolocation information through soil microbiome, and making use of thanato-microbiome and epinecrotic microbial communities to estimate postmortem intervals are a few examples. With its many benefits over conventional microbiological techniques, massive parallel sequencing makes it possible to detect and analyse microbial communities with previously unheard-of speed and accuracy. To guarantee dependability, reproducibility, and effective data analysis, incorporating MPS in forensic contexts calls for the creation of established methods, extensive reference libraries, and improved computational tools.**

The ongoing advancement of MPS technology has enormous potential to enhance forensic procedures and results. Continuous improvements in MPS technology have the potential to completely transform forensic procedures and improve the precision, effectiveness, and results of forensic investigations. MPS technology is an essential instrument in the forensic science toolbox, and as it advances, we may expect significant advancements in forensic procedures and the handling of challenging cases.

Keywords— **Microbial Forensics, Massive Parallel Sequencing (MPS), Thanato-microbiome, Human Identification, Postmortem Interval (PMI).**

I. INTRODUCTION

Today's forensic science discipline is seeing new methods to criminal investigations thanks to the convergence of biological discoveries and technological advancements [1]. Microbial forensics is one such cutting-edge technique that uncovers crucial forensic information by utilizing the diversity and richness of microbial communities. Utilizing the expanding capabilities of bioinformatics and recent advancements in the generation of genetic data, particularly through massive parallel sequencing (MPS), this multidisciplinary field emerged from the nexus of microbiology and forensic research.[2]

The initial applications of microbial forensics were in specialist domains such as bioterrorism, bio crime, and epidemiology. These early applications demonstrated the usefulness of microbiological evidence in identifying and monitoring biological dangers. However, the discipline of microbiological forensics has expanded greatly since then, taking use of many new opportunities. These include using the microbiomes of skin, hair, and bodily fluids to help identify people; using the soil microbiome to provide geolocation information; using the thanato-microbiome and epinecrotic microbial communities to determine postmortem intervals (PMI); using microorganisms as ancillary evidence in criminal cases; and elucidating the causes of death in a range of situations (e.g., drownings, toxicology, hospital-acquired infections, and SIDS) [3].

The application of MPS in microbial forensics offers numerous advantages over traditional microbiological methods. It enables the detailed and high-throughput analysis of microbial communities, providing a comprehensive view of the microbial landscape in forensic samples. MPS facilitates the identification of microbial signatures with greater accuracy and speed, making it an invaluable tool in forensic investigations. Despite these advantages, the integration of MPS in forensic contexts necessitates the development of standardized protocols and the establishment of robust reference databases to ensure the reliability and reproducibility of results. This underscores the need for concerted efforts in the forensic community to formulate guidelines and standards that will support the widespread adoption of MPS in forensic applications. [4]

However, since many microorganisms are not culturable and quick results are crucial in forensic settings, culturing is inadequate.[5] Culturing provides identification at the genus/species level, but it is not detailed enough for forensic

attribution. Degraded agents and restricted quantities are common problems with forensic samples.[6] The goal of microbial forensics is to accurately identify individual isolates by utilizing nucleic acid analysis to reliably link or exclude sources. Like human DNA forensic analysis, this method improves forensic investigations by offering comprehensive microbiological data that is necessary for precise attribution. [7] Nucleic acid-based analyses are becoming more and more accessible for clinical diagnosis and are required in microbial forensics. These methods improve quick diagnosis and sample source identification, but they are not a panacea. In addition to exploring technologies that enhance the microbiological forensics toolkit, this review looks at genetic concerns that impact the interpretation of forensic evidence [8].

II. LITERATURE REVIEW

Because it can create links between people, things, and crime scenes, trace evidence is crucial to forensic science. Trace evidence is defined as the remaining proof of an incident and comprises a variety of items discovered at crime scenes. One of the first forensic scientists to use microbes as trace evidence was Alphonse Bertillon, who examined their origins at several locations throughout Paris. Six fundamental concepts are involved in forensic analysis, according to Inman and Rudin's model: reconstruction, identification, association, categorization, transfer, and divisibility. This comprehensive approach provides valuable insights into criminal investigations.[9]A significant threat to society is the outbreak of serious diseases, whether naturally occurring or intentional. In addition to causing disruption, panic, and harm or death, these incidents can also influence the economy. New tools to fight reemerging and emerging diseases have been made possible by developments in pathogen and molecular biology.

However, these advancements can also be misused to create biological weapons for terrorism, causing severe harm to humans, animals, and plants. Pathogens or toxins, used to commit acts of terror, can also serve as weapons in crimes. Bio crimes use biological weapons rather than traditional ones like guns or knives, much like traditional crimes that target specific individuals. Bioweapons are instruments for retaliation, extortion, or personal vengeance that can be used for murder or physical harm. Advances in molecular biology combined with forensic science highlight the value of trace evidence, particularly in criminal cases. The pioneering use of microbes as trace evidence by Alphonse Bertillon demonstrates how forensic techniques can adapt to modern dangers.

The systematic process of forensic analysis, which includes divisibility, transfer, categorization, identification, association, and reconstruction, is still essential for assembling crime scenes and connecting evidence to illegal activity. [10] Because microorganisms are so common, bacteriology and mycology are now essential components of forensic procedures like toxicology and thanatology. These are necessary supplemental evidence, but they are not primary approaches. The fields of forensic microbiology, commonly referred to as post-mortem microbiology, are concerned with the epinecrotic community (microorganisms on decomposing bodies) and the thanatomicrobiome (microorganisms in the body after death). Even though post-mortem microbiome research is fraught with difficulties and debates, these studies provide important new information. The necro- and thanato-microbiomes' potential as forensic tools to improve crime scene comprehension and identify reasons of death is highlighted in this review of forensic microbiology research. [11]

III. METHODOLOGY

Identification and description of microbes have been greatly impacted by technologies that can analyze genetic markers for phylogenetic inference, especially nucleic acid amplification technology. [12] Hybridization and restriction fragment length polymorphism (RFLP) typing were labor-intensive methods used for nucleic acid-based microbial identification prior to Saiki et al.'s 1985 invention of the polymerase chain reaction (PCR). These techniques were impractical for forensic investigations because they needed a lot of nucleic acids, took a long time, and could not be automated.[13] The field was completely transformed with the advent of sequence-specific amplification tools, which offered a wide variety of methods specifically designed to meet the demands of the microbiological forensic community.

PCR enabled the rapid amplification of specific DNA sequences, allowing detailed analysis of small and degraded samples previously challenging to work with.[14] This innovation increased the sensitivity and specificity of microbial detection and identification, making it possible to analyse even trace amounts of microbial DNA from forensic samples. Nucleic acid amplification technology, including real-time PCR, multiplex PCR, and next-generation sequencing (NGS), has significantly enhanced microbial forensics. Real-time PCR quantifies DNA, providing valuable information about microorganism concentrations in a sample. Multiplex PCR allows simultaneous amplification of multiple genetic targets, increasing efficiency and throughput. NGS offers a comprehensive view of the microbial community in a sample, identifying a wide range of microorganisms with unprecedented resolution. Integrating these technologies into forensic workflows has transformed microbial forensics, enabling rapid and accurate detection and characterization of microorganisms.[15] This has significant implications for forensic investigations, enabling pathogen identification, determining the origin of microbial evidence, and excluding unrelated sources. The forensic community must adopt new initiatives and

standardized protocols to fully exploit nucleic acid-based analyses, enhancing the accuracy and reliability of microbial forensic investigations.[16]

i. PCR (A sequence-specific amplification tool)

CR-based methods are favored in molecular biology due to their simplicity, requiring minimal genomic DNA and applying to non-viable organisms or direct clinical specimens. PCR, the cornerstone of modern molecular assays, functions in vitro to exponentially amplify targeted DNA sequences. The process, both theoretically and practically simple, necessitates specific reagents: a nucleic acid template, oligonucleotide primers, deoxynucleotide triphosphates, a buffer, a cation like magnesium, and a thermostable DNA polymerase.[17]

Theoretically and practically straightforward, the procedure requires certain materials, including a nucleic acid template, oligonucleotide primers, deoxynucleotide triphosphates, a buffer, a cation such as magnesium, and a thermostable DNA polymerase.[17]

A thermal cycler, which carefully regulates temperature cycles to aid in the PCR process, holds a reaction tube containing all of the ingredients. Annealing of the oligonucleotide primers bordering the target DNA segment, extension of the annealed primers, and denaturation comprise a typical PCR cycle. A key role is played by primers, which are brief single-stranded oligonucleotides of roughly 20 to 30 base pairs. The target DNA, extracted from a sample or synthesized via reverse transcription, is first denatured by heating to 95°C. This step separates the DNA strands, allowing primers to bind (hybridize) through complementary base pairing when the temperature is lowered to an empirically determined range, typically between 45°C and 65°C. The subsequent phase, primer extension, usually occurs at 72°C. At this temperature, Taq polymerase, the most used enzyme in PCR, synthesizes complementary DNA strands from the primers, effectively doubling the amount of target DNA. This exponential amplification is due to newly synthesized DNA fragments serving as templates in subsequent cycles.[18] Typically, 25 to 45 cycles are performed, generating millions to billions of copies of the target sequence within 30 minutes to two hours. PCR's development marked a significant advancement over earlier techniques like hybridization and restriction fragment length polymorphism (RFLP) typing, which were labor-intensive, required large nucleic acid, and lacked automation potential. PCR's ease and efficiency have revolutionized microbial detection and characterization, facilitating rapid, sensitive, and specific analysis of even minute and degraded samples.[19] PCR-based technologies such as real-time PCR, multiplex PCR, and next-generation sequencing (NGS) have further enhanced microbial forensics.[Table 3.1] Real-time PCR quantifies DNA, providing insights into microorganism concentrations in samples. Multiplex PCR allows simultaneous amplification of multiple genetic targets, increasing analytical efficiency. NGS offers a comprehensive view of microbial communities, identifying a broad array of microorganisms with unprecedented resolution.[20] Integrating these technologies into forensic workflows has transformed microbial forensics, enabling swift and accurate microorganism detection and characterization. These capabilities are crucial in forensic contexts where rapid results are imperative to support ongoing investigations.[21] The specificity and sensitivity of PCR-based methods have expanded the range of detectable microorganisms, providing forensic scientists with powerful tools to trace microbial evidence to specific sources or exclude potential sources with high confidence.[22] However, the effective application of PCR in forensics requires careful consideration of various factors. The quality and quantity of the sample are paramount, as forensic samples are often limited and may be degraded. Ensuring the integrity and viability of nucleic acids is essential for accurate analysis. Moreover, the establishment of robust reference databases is crucial for interpreting genetic data and achieving reliable forensic attributions.[23]

ii. Assay-based Hybridisation

After the PCR process, DNA marker alleles are identified using various analytical techniques, primarily based on the hybridization of probes or primers and electrophoresis. Research by Woese et al. (1985, 1987, 2000) highlighted that phylogenetic relationships among bacteria and all life forms can be determined by examining genetically conserved regions of the genome.[24] Among the most informative gene sets for this purpose are the ribosomal RNA genes, including the 5S, 16S, and 23S rRNA genes, along with intergenic spacer regions. The 16S rRNA gene is often utilized for phylogenetic positioning and speciation because of its conserved and slowly changing sections. It is critical for cellular function; thus, mutations are rare, enhancing its utility in fine-grained taxonomic analysis.[25] PCR amplifies the 16S rRNA gene or its segments, with clinical diagnostics often focusing on ~500 bp fragments that provide sufficient discriminatory power and are easy to sequence. The sequenced amplified products are then compared against databases such as GenBank and the Ribosome Database Project. [Table 3.1] Commercial databases like the MicroSeq (Microbial Identification System) also offer extensive data, including sequences from over 1,400 microorganisms.[26] When an exact match is unavailable, algorithms position the sample data relative to known taxonomies. PCR amplifies the 16S rRNA gene or its segments, with clinical diagnostics often focusing on ~500 bp fragments that provide sufficient discriminatory power and are easy to sequence. The sequenced amplified products are then compared against databases such as GenBank and the Ribosome Database Project.[27] Commercial databases like the MicroSeq Microbial

Identification System also offer extensive data, including sequences from over 1,400 microorganisms. When an exact match is unavailable, algorithms position the sample data relative to known taxonomies. Despite the utility of 16S rRNA gene typing, its resolution is limited by the breadth and depth of available databases[28]. For higher resolution, the 16S-23S rRNA gene internal transcribed spacer (ITS) can be sequenced. Unfortunately, no common gene in viruses allows for such speciation, whereas fungal speciation often relies on the 18S rRNA gene, typically limited to genus or family levels. The ITS region between the 18S and 28S rRNA genes provides higher resolution due to its faster mutation rate, enhancing species differentiation.[29]

By examining internal regions of many housekeeping genes that are shared by numerous bacteria, multilocus sequence typing (MLST) provides an additional technique [Table 3.1].

Public MLST databases facilitate comparisons across numerous species, aiding in studying recombination and its genetic diversity impact.[30] MLVA (multi-locus VNTR analysis) amplifies DNA fragments differing in repeat segment numbers, providing strain-level resolution in bacteria with repeat elements. However, its reliance on high-mutation-rate sites limits its utility in lineage-based studies. SNP analysis using systems like the ABI PRISM SNaPshot system improves hybridization kinetics and typing specificity, offering robust methodologies for microbial forensic analysis.[31] In summary, post-PCR analytical techniques such as 16S rRNA gene typing, MLST, MLVA, and SNP [Table 3.1] analysis play crucial roles in microbial forensics, providing detailed genetic data essential for accurately identifying and characterising microbial samples in forensic investigations.[32]

Table 3.1 Techniques Applied in the Amplification of Gene

Technique	Description	Utility	Limitation
16S rRNA	Amplifies and sequences the 16S rRNA gene (~500 bp fragments). Compared against databases like GenBank and the Ribosome Database Project.	Extensively used for phylogenetic positioning and speciation due to conserved regions.	Limited resolution due to the breadth and depth of available databases.
16S-23S rRNA	Sequences of the ITS region between the 16S and 23S rRNA genes.	Provides higher resolution and species differentiation due to faster mutation rates.	No common gene in viruses allows for such speciation; fungal speciation is often limited to genus or family levels.
Multilocus Sequence Typing (MLST)	Internal segments of many housekeeping genes that are common to various bacteria are analyzed by Multilocus Sequence Typing (MLST).	Provides unique characterizations of bacterial species and distinguishes many strains within species.	Relies on standardized databases; limited by availability and scope of public MLST data.
Multi-Locus VNTR Analysis (MLVA)	Amplifies DNA fragments differing in repeat segment numbers.	Provides strain-level resolution in bacteria with repeat elements.	Relies on high-mutation-rate sites, limiting utility in lineage-based studies.
SNP Analysis (ABI PRISM SNaPshot System)	Uses primer hybridization and electrophoresis for multiplex SNP analysis.	Improves hybridization kinetics and typing specificity; robust for microbial forensic analysis.	Requires advanced technology and computational tools.

IV. APPLICATIONS OF MICROBIOLOGY IN FORENSICS

Microbes play a vital role in determining the cause and manner of death. Researchers have examined microbial communities from different bodies to find biomarkers that could aid in forensic investigations. While promising, these studies are limited by sample size and the need for extensive databases. Identifying a single bacterial species in cadaveric material usually indicates an infection, whereas mixed species suggest postmortem contamination.[33] Accurate

postmortem microbial assessments require quantitative evaluations to establish the significance of microbial presence. This is crucial for demonstrating a direct link between microbial isolation and death. Histological studies, paired with microbiological data, can assist distinguish between infections contracted before death and postmortem contamination. Factors such as the transmission of bacteria during the dying process, the time between death and autopsy, and prior manipulations can affect the reliability of microbiological findings. The use of antibiotics during life complicates postmortem microbiology (PMM), as they may not eradicate infections, leaving the immune system to manage them.[34]

i. In cases like **Sudden Infant Death Syndrome (SIDS),** comprehensive microbiological examinations are essential to investigate unexplained deaths. Identifying bacterial or viral infections in such cases can be challenging, but it remains crucial for understanding potential causes of death.[35]

ii. Sepsis

Post-mortem diagnosis of sepsis is challenging due to nonspecific clinical and microscopic findings and potential contamination of cultures. Bacterial infections are the most prevalent cause, but fungal, viral, and protozoal infections can also cause sepsis. Recent approaches integrate cultural, microscopic, biochemical, and immunohistochemical analyses.[36]

iii. Food Toxin Infection

Food poisoning deaths occur when food or water is contaminated with bacteria, viruses, fungus, parasites, or poisons. Salmonella, Campylobacter, and E. coli are among the most often found bacteria. They act by invading the intestine or producing toxins. Diagnosis involves autopsy and microbiological studies, with molecular diagnostics increasingly important.[37]\

iv. Other Infectious Diseases

Microbiological examinations are invaluable in diagnosing diseases like pneumonia, mycobacterial infections, fungal infections, infective endocarditis, and meningitis post-mortem. Pneumonia is often diagnosed first at autopsy, identifying resistant microorganisms. Tuberculosis diagnosis varies by region and is confirmed through culture.[38] Fungal infections, common in immunocompromised patients, are often undiagnosed in vivo but identified post-mortem. Infective endocarditis, often missed in life, is detected through autopsy of affected valves. Meningitis, typically diagnosed in hospitals, can cause sudden death, and requires microbiological examination to identify the pathogen. These examinations help confirm causes of death and inform public health strategies.[39]

v. Death by Drowning

Forensic diagnosis of drowning involves confirming data through laboratory analysis after excluding other causes of death. Traditionally, diatoms are used as indicators, but their reliability is debated. Studies now focus on bacterioplankton as an alternative. Samples from the liver, kidney, lungs, and blood are tested using a molecular PCR assay with fluorophore-labelled TaqMan probes. This technique detects bacterial components in both freshwater (hypotonic) and seawater (hypertonic) drowning cases, identifying species like Aeromonas, Plesiomonas shigelloides, Vibrio, and Photobacterium.[40]

V.CONCLUSION

A number of inquiries can be resolved by genetic-based analysis, which include identifying the type and origin of the evidentiary sample, evaluating its exculpatory or probative value, and analyzing correlations between questioned and known samples for attributional purposes.[41] Molecular assays that facilitate sample attribution and source traceability are made possible in large part by nucleic acid-based technology.[42] Due to space limitations, certain methods for microbial genetic analysis are not discussed here, such as mass spectrometry-based Triangulation Identification for Genetic Evaluation of Risks (TIGER), fluorescent in situ hybridization (FISH), and rep-PCR.[43] These analytical skills enable both qualitative and quantitative evaluations when paired with preexisting facts and assumptions. Inquiries and resources can be resolved by genetic-based analysis, which include identifying the type and origin of the evidentiary sample, evaluating its exculpatory or probative value, and analyzing correlations between questioned and known samples for attributional purposes.[41] Molecular assays that facilitate sample attribution and source traceability are made possible in large part by nucleic acid-based technology.[42] Due to space limitations, certain methods for microbial genetic analysis are not discussed here, such as mass spectrometry-based Triangulation Identification for Genetic Evaluation of Risks (TIGER), fluorescent in situ hybridization (FISH), and rep-PCR.[43] Both qualitative and quantitative evaluations of microbiological forensic tests are made possible by these analytical capabilities in conjunction with the data and presumptions now in existence. However, a better understanding of genetic variation and population dynamics, as well as enhanced computational methods, are necessary to improve trust in forensic interpretations, particularly regarding statistical confidence in the weight of the evidence.[44] There are several opportunities to improve the understanding and analytical skills required for evaluating microbial forensic genetic evidence. Numerous potentials for the microbiological forensic community necessitate extending research on microorganisms at the species, population, and strain levels as well as evaluating their

inherent variability.[45] Finding genetic fingerprints for attribution and recognizing manufactured infections require improved detection capabilities. For gathering, managing, preserving, preparing, concentrating, and extracting samples. The threat of bioterrorism and bio crime remains since microorganisms are widely available and creating and distributing bioweapons is extremely inexpensive.[47] With the help of national and international resources, scientists from federal agencies, state institutions, universities, and private businesses will need to work together to address these issues.

This review aims to guide and encourage those interested in contributing to the growing field of microbial forensics, highlighting the importance of developing robust methodologies and tools for forensic investigations.[48] Microorganisms have long been pivotal in forensic evidence, and as microbiology evolves, new applications are emerging with great potential. While some applications are more developed, many are still in preliminary stages.[49] This review compiles current forensic microbiology applications to offer a comprehensive overview. Microbiological studies are crucial in autopsy examinations, and further research is necessary to fully explore their forensic potential. However, microbiology is undoubtedly a fundamental discipline in forensic investigations.[50]

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