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Review

Sequencing Technologies in Forensic Microbiology: Current Trends and Advancements

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Abstract: Forensic microbiology is a subject of interest and research development across the forensic community. Studies of pathogen outbreaks, biocrime or bioterrorism attacks, and analyses of crime scenes fall within this field. Significant progress has been made in evolving DNA sequencing technologies in recent decades. These newly emerged transformative tools have become available to both biomedicine and forensics. Based on the published literature, this review provides an overview of the current trends and developments of NGS (next-generation sequencing) technologies applied to forensic microbiology. These new methods present numerous advantages over traditional sequencing technology and are useful in several practical applications within this forensic field. This article then examines the main challenges and limitations of this technology in forensics, providing a comprehensive review of NGS technology capabilities in expanding the precision and effectiveness of microbial forensic investigations—with the aim of inspiring scientists, forensic experts, lawyers, public health professionals, and policymakers alike to approach this newly powerful sequencing tool appropriately.

Keywords: bioterrorism; crime/death scene; epidemiology; forensic applications; microbial communities; microbial forensics; next-generation sequencing

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1. Introduction

Forensic microbiology, a discipline that emerged from the intersection between microbiology and forensic science, offers applications in multiple forensic scenarios—from crimes involving bioterrorism and biocrimes, understanding the dynamics of infectious outbreaks, unraveling the origins of microbial contamination, and resolving civil and criminal cases [1–3]. In its essence, forensic microbiology profits from the power of scientific investigation to decipher the often silent but substantial testimonies of microorganisms (e.g., bacteria, viruses, fungi, and parasites), providing insights with comprehensive implications for public health, biosecurity, and criminal justice [4,5]. However, as proposed by Tsokos and Püschel's study [6], analyzing the changes in microorganisms' composition is challenging. For instance, post-mortem translocation of bacteria across different body parts after death can complicate the analysis, resulting in misleading results. Nevertheless, the same study proposes methodological approaches to mitigate this risk (e.g., aseptic techniques).

Over the last few years, forensic microbiology has undergone a transformative revolution instigated by the advancements in DNA sequencing technologies that pave the way for a new era of molecular investigation [7,8]. Among these advancements, next-generation sequencing (NGS) has revolutionized the understanding of the microbial world. Characterized by its high-throughput capabilities, rapid sequencing speeds, and ability to generate massive amounts of data in a single sequencing run [9,10], NGS offers scientists, forensic experts, and public health practitioners unprecedented insight into the composition of diverse microbial communities.

By reviewing published data, this manuscript explores the relationship between NGS technology and forensic microbiology, aiming to elucidate the current trends and advancements. Therefore, the applications, advantages, and challenges of NGS in forensic microbiology investigations are examined, guided by the understanding that genomics is essential for storytelling in microbial life.

The manuscript selection process in the present revision followed a structured approach with precise inclusion and exclusion criteria. More than 15,000 manuscripts were retrieved from public article databases, such as PubMed (U.S. National Library of Medicine, Bethesda, USA), Web of Science, Scopus, and Google Scholar, using the keywords "Forensic Microbiology", "Next-generation Sequencing"; "Forensic Applications"; "Advantages"; "Limitations". Due to the novelty of the topic, no date limit was applied. Only peer-reviewed articles with robust methods and high-quality data were considered as inclusion criteria (journals belonging to Q1 and Q2). Manuscripts that demonstrated practical relevance, showing clear applications to casework scenarios, were also included. As exclusion criteria, manuscripts that did not directly address the core topics, lacked scientific rigor, or were outdated were removed from the analysis. This evaluation was based on Abstract analysis. After this selection process, the research team analyzed the complete length of the more than 500 manuscripts. The process ensured that the revised manuscript incorporated the most pertinent and high-quality research, providing a comprehensive update on forensic microbiology.

The present manuscript has been divided into six sections. The first section introduces the concept of forensic microbiology as an intersection of microbiology, genetics, and forensic sciences to investigate microbial agents. This section also explains how microorganisms leave genetic traces that can reveal their source, identity, and behavior, aiding in criminal investigations, public health emergencies, environmental incidents, and bioterrorism attacks. The section Next-Generation Sequencing (NGS) Technology covers the evolution of sequencing technologies from first-generation sequencing (FGS) to second-generation sequencing (SGS) and Third-Generation Sequencing (TGS). Readers will be elucidated on the advancements that have enhanced genetic analysis precision, speed, and scale. The following section details NGS applications such as pathogen identification, epidemiological investigations, source attribution in outbreaks, biodefense, and forensic genomics. The section Advantages of Next-Generation Sequencing Technology in Forensic Microbiology details the benefits of NGS, such as detecting microorganisms at trace concentrations,

identifying a broad spectrum of pathogens, faster turnaround times, and detailed genomic characterization, which are valuable for forensic investigations and court evidence. The following section, Limitations of Next-Generation Sequencing Technology in Forensic Microbiology, points out the main challenges associated with this technology, such as the quality of post-mortem samples, environmental contamination, the need for comprehensive databases, complex bioinformatics analysis, and high costs. The manuscript ends with a Conclusion section that summarizes the main findings of this manuscript and points out innovative approaches (i.e., including k-mer-based techniques) and further research topics (i.e., the introduction of artificial intelligence in forensic microbiology).

2. Forensic Microbiology

Forensic microbiology, a dynamic and multidisciplinary field, applies the principles of microbiology, genetics, and forensic science to investigate and analyze microbial agents. These investigations embrace several purposes, such as criminal investigations, public health emergencies, environmental incidents, and bioterrorism threats [11–13]. In a very simplified approach, forensic microbiology intends to answer critical questions, such as "Who?", "What?", "When?", "Where?" and "Why?", associated with microbial events and unveiling microorganisms' diversity and their societal impact [14,15].

This science is founded on the principle that all microorganisms leave traces of their presence, whether in infected patients, contaminated samples, or environmental reservoirs, along with genetic information that can be analyzed for investigative purposes [16–19]. Therefore, forensic microbiology deciphers the genetic variations within these traces, revealing vital information about the microorganisms' source, identity, and behavior [17,20,21].

The origins of forensic microbiology can be traced back to the pioneer works in microbiology, such as those of Louis Pasteur or Robert Koch, who laid the groundwork for this field. Pasteur's contributions include sterilization, vaccination, and pasteurization, while Koch contributed with the postulates and disease-causing bacteria identification [22]. The basis for modern forensic microbiology emerged several years later, in the late 20th century, with the development of classical DNA sequencing techniques, such as the Polymerase Chain Reaction (PCR), which offered new possibilities for the accurate and rapid identification and characterization of microbial agents [23].

Forensic microbiology presents diverse applications dispersed across several scenarios [2,9]:

(a) In public health and epidemiology: identification of the pathogens responsible for disease outbreaks, traces of their sources and transmission pathways, and implementation of containment measures to protect public health. The inclusion of these aspects as applications of forensic microbiology is a matter of debate by many authors. However, public health and epidemiology share the same objectives and methods as forensic cases, resulting in microorganism isolation and source attribution.
(b) In biocrime and bioterrorism: determination of the source of the harmful pathogen releases and attribution to specific individuals or groups.
(c) In environmental incidents (e.g., water supply contamination or foodborne outbreaks): identification of the agents involved, tracking their origins and transmission pathways, implementing containment measures, and preventing future incidents.
(d) In criminal investigations: analysis of microbial evidence from crime scenes (e.g., microbial communities associated with decomposing bodies, soil samples, or biological fluids) that provides valuable clues for case resolution.

3. Next-Generation Sequencing Technology

The developments in NGS technologies initiated a transformative era in genetics and genomics, allowing researchers to explore genetic variation at a deeper level and with unprecedented precision, velocity, and scale [24–26].

Based on the traditional Sanger sequencing [27], NGS technology surpasses the limitations of its predecessor (i.e., time and labor-intensive, and limited scalability) [28–30]. NGS uses high-throughput techniques to sequence multiple DNA fragments simultaneously,

producing massive amounts of sequencing data in a single run. Consequently, this technology makes large-scale sequencing projects more cost-effective and accessible to a broader research population compared to more conventional alternatives [31,32].

Conventionally, NGS comprehends several sequential steps, including the following [33,34]:

(a) DNA extraction (specific methods are required to ensure efficient DNA extraction);
(b) Library preparation (nucleic acid samples are fragmented and tagged with unique identifiers);
(c) Sequencing (samples are loaded onto an NGS platform and tagged nucleic acid molecules are sequenced) and data generation (reads production);
(d) Data analysis (bioinformatics tools and computational pipelines process and analyze sequencing data);
(e) Data interpretation (identification of genetic variations, mutations, or other relevant information within the genome).

3.1. First-Generation Sequencing Technology

First-generation sequencing (FGS) involves the incorporation of chain-terminating nucleotides—dideoxynucleotides (ddNTPs)—into growing DNA strands [27,35,36]. Applied Biosystems ABI 370, the first automated sequencing machine, became available in the late 1980s [37], being followed by the ABI310 which uses fluorescently labeled ddNTPs and capillary electrophoresis, increasing sequencing speed and accuracy [38,39].

This new technology was applied to the Human Genome Project (HGP), an international consortium that sequenced and mapped the human genome; the project was completed in 2003 and constituted a milestone in human genetics. This method is also an indispensable tool in molecular biology, elucidating the location and functions of genes and regulatory regions and the genetic basis of diseases. Finally, in genetic diagnostics, FGS identifies disease-causing mutations, contributing to the development of personalized medicine [40,41].

Although surpassed by more modern technologies, the FGS legacy still endures since this technique remains an invaluable tool in molecular biology and diagnostics, particularly in verifying and validating DNA sequences generated by NGS platforms.

3.2. Second-Generation Sequencing Technology

Based on FGS and introducing the innovative possibility to sequence millions of DNA fragments in a single run simultaneously, second-generation sequencing (SGS) was developed, offering high-throughput, cost-effective, and rapid DNA sequencing, and becoming the backbone of modern genomics [42].

Developed in the mid-2000s, SGS enables a wide range of applications, such as whole-genome sequencing, targeted sequencing, metagenomics, and transcriptomics, among others [43,44]. Since its invention, several platforms have become commercially available. Among them, Roche 454 Pyrosequencing is based on the detection of the release of pyrophosphate during DNA synthesis, producing longer read lengths (up to 700–800 base pairs) with lower throughput capacity (approximately 400 Mb per run) when compared to other technologies. Ion Torrent sequencing measures changes in pH as nucleotides are incorporated into a growing DNA strand. It offers a rapid sequencing protocol, particularly suited for targeted sequencing applications (up to 400–600 bp, but recent improvements have aimed for longer reads; throughput capacity: up to 10 GB, depending on the platform model) [45,46]. Illumina technology, a cornerstone of SGS, immobilizes DNA fragments onto a solid surface, followed by iterative cycles of nucleotide incorporation and fluorescence-based detection, generating short-read sequences (usually 150–300 bp, with recent advancements enabling up to 600 bp in some configurations) with high throughput capacity and accuracy (ranges from 1 to 6 Tb per run, depending on the instrument model) [47]. Applied Biosystems' SOLiD (Sequencing by Oligonucleotide Ligation and Detection) technology employs a unique ligation-based approach to sequencing that offers

high accuracy (sequence read length: typically, 50–75 bp; throughput capacity: up to 1–2 Gb per run, depending on the system version) [48].

Depending on the molecule and the sequencing approach, SGS provides different results. In whole-genome sequencing, entire genomes are sequenced, providing information on genetic variation, evolution, and the genetic basis of diseases [49]. In transcriptomics, RNA sequencing (RNA-Seq) allows gene expression to be studied, alternative splicing, and non-coding RNA, unraveling gene regulatory networks [50]. In epigenomics, it enables profiling DNA methylation patterns, shedding light on epigenetic regulation [51]. In metagenomics, complex microbial communities from diverse environments, from the human gut to extreme ecosystems, can be analyzed [52]. In targeted sequencing, specific genomic regions of interest, such as exomes or disease-associated genes, can be selectively sequenced [53]. In clinical diagnostics, it identifies disease-causing mutations, hereditary conditions, and pharmacogenomics.

3.3. Third-Generation Sequencing Technology

Third-generation sequencing (TGS) technology was developed to overcome the limitations of the previous technologies, promising to revolutionize genomics research and clinical diagnostics, among other fields. These platforms, which emerged in the 2010s, offer unprecedented capabilities in read length, speed, and the ability to observe DNA molecules in real time [54–56].

Several TGS platforms have been developed with an independent sequencing approach. Among them, PacBio Sequencing (Pacific Biosciences) allows for single-molecule real-time (SMRT) sequencing and employs zero-mode waveguides (ZMWs) to monitor the incorporation of individual nucleotides, producing long reads and enabling the sequencing of complex genomic regions and identification of structural variations [57]. Oxford Nanopore Sequencing uses nanopores (i.e., small protein channels) to read DNA strands as they pass directly through the pore, offering long reads and portability for field applications and rapid diagnostics. Nanopore Sequencing (Sequel II System) offers a combination of short-read and long-read sequencing capabilities [58].

TGS technologies provide numerous possibilities in genomics research, such as de novo genome assembly (assembling complex genomes without the need for a reference genome), detection of structural variations (e.g., inversions, and complex genomic rearrangements), epigenomics (e.g., DNA methylation, histone modifications), RNA sequencing (e.g., alternative splicing, isoform diversity, and transcript isoform identification), clinical diagnostics (accurate detection of rare genetic variants and structural abnormalities), and metagenomics (e.g., characterization of complex microbial communities, revealing novel species and functional potentials) [59–61].

4. Applications of Next-Generation Sequencing Technology to Forensic Microbiology

Next-generation sequencing technology has the potential to revolutionize forensic microbiology, enabling researchers to have the ability to decipher microorganisms' genetic composition with unparalleled turnaround time, accuracy, and depth, making it invaluable for various applications [24].

The two predominant methods for microbial identification using high-throughput data analysis are targeted amplicon-based sequencing and whole metagenome sequencing (WMS).

Targeted amplicon-based sequencing involves amplifying specific, informative genome regions that serve as markers for understanding microbial diversity and relationships within microbial communities. Depending on the microorganisms being studied, the most used genomic regions are 16S ribosomal RNA and the internal transcribed spacer (ITS) region. The 16S rRNA gene, central to bacterial identification, contains highly conserved regions that serve as primer regions, and several variable regions (V1-V9) that allow discriminating between microbial groups [62]. The sequencing of this gene allows the deciphering of bacterial taxonomy and phylogeny: however, its resolution at the infra-species level can

be restricted [63], requiring the analysis of other genomic regions. The ITS region for fungal identification contains the non-coding segments between rRNA genes. Its sequencing offers precise species-level recognition due to high sequence variation levels [64].

Although all previously presented advantages of targeted amplicon-based sequencing, this method also presents challenges. For instance, the taxonomic biases associated with choosing primers [65] or amplified regions [66] are well known. Furthermore, the amplification process can result in chimeric sequences [67] that contain 16S rRNA pieces from more than one organism.

On the other hand, WMS sequencing goes beyond targeted regions to sequence the entire metagenome, uncovering comprehensive genomic data and subtle genetic variations. WMS sequencing involves extracting DNA from a sample without targeting a specific region or amplifying it before sequencing. This approach differs from the targeted marker amplification method by directly providing taxonomic and functional information from the data. It offers the possibility of de novo assembly of microbial genomes, enabling the exploration of previously unknown species.

Additionally, both prokaryotic and eukaryotic DNA can be analyzed simultaneously in this process [68]. Nevertheless, the WMS sequencing approach produces vast data, necessitating adequate storage and computational capabilities. Moreover, attaining resolution at the strain level often demands extensive sequencing, which can substantially raise expenses [69].

4.1. Pathogen Identification and Characterization

NGS enables comprehensive genome sequencing from diverse microorganisms such as bacteria, viruses, fungi, and parasites. Results provide complete genetic profiles that accurately differentiate between species and even closely related sub-species strains, without compromising turnaround time or accuracy. This comprehensive approach is useful in complex samples where traditional methods may fall short, such as environmental or clinical specimens, being particularly suitable in cases of a disease outbreak (Section 4.2) or a bioterrorism event (Section 4.3). Sequencing the genomes of the microorganisms involved allows researchers to pinpoint the specific strain or variant responsible, which is critical to establishing effective containment and response strategies [70–72]. Moreover, NGS is culture-independent, allowing for the sequencing of all genetic material without the need to grow pathogens, which is a particular aspect since several important pathogens do not grow in standard culture media. This capability also allows for rapid outbreak detection, since pathogens can be sequenced from multiple sources, time-effectively identified, and track the source of outbreaks, facilitating timely public health responses [73–75].

NGS excels in pathogens characterization, analyzing genetic variations within pathogen genomes, and providing insights into pathogen evolution, resistance mechanisms, and virulence factors. Moreover, NGS allows for antibiotic resistance profiling by identifying genetic elements associated with resistance, which informs treatment strategies. It enables detailed monitoring of genetic changes, including mutations and recombination events for viruses, which is essential for tracking viral evolution and evaluating vaccine effectiveness [73,76,77].

This technique has been applied to pathogen identification and characterization. In the manuscript by Sjödin and collaborators [78], the authors highlight the importance of NGS in either epidemiological or bioterrorism attacks and call attention to the need for more comprehensive genomic databases while explaining the difference between epidemiological investigations (comprising the identification of the biological agent(s) responsible for an event—identification); and characterization of the event as either intentional or unintentional—characterization) and bioterror attacks (comprising the two previous steps and adding the identification of the person(s) responsible for the attack—attribution). On the other hand, Turner and collaborators [79] refer to the importance of NGS and bioinformatics technologies in discriminating the causes of infectious disease outbreaks (i.e., natural, accidental, and deliberate).

4.2. Epidemiological Investigations

Pathogens have rapidly evolved, and the analysis of genetic variations, such as single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), and gene rearrangements, through the use of NGS, can reveal the evolutionary relationships between different strains, helping to trace the origin and spread of an outbreak [72,78–80]. In fact, in outbreaks (e.g., foodborne pathogens or emerging viruses), these technologies can monitor pathogen evolution and transmission in near real time, providing invaluable data to inform containment strategies [81,82]. Moreover, as previously referred, antibiotic resistance genes and virulence factors are critical for understanding the outbreak's potential impact and guiding public health interventions. By uncovering the genetic basis of these traits, NGS can inform treatment strategies and help prevent the spread of resistant or particularly virulent strains [73,77].

In clinical microbiology, this technology can assist in designing diagnostic and genotyping tools, identifying virulence and antibiotic-resistance mechanisms, and developing specific culture media [83,84]. It has been proposed that, shortly, NGS will replace many traditional microbiological workflows in clinical diagnostics and public health surveillance [85].

NGS emerged as a powerful tool for identifying and tracking hospital-acquired infections (HAIs). This technology can characterize the hospital environment's microbiome, surpassing traditional methods in sensitivity and enabling the detection of previously unrecognized bacteria, identify antibiotic resistance genes and multi-drug-resistant infections, and distinguish between single-source and multiple-source outbreaks, preventing unnecessary infections [86–89].

4.3. Source Attribution

Traditional methods for microbial source attribution (e.g., phenotypic analysis, basic genetic typing) often lack the resolution needed to distinguish between closely related strains or to track the possible transmission routes [90]. However, due to its capacity to sequence entire microbial genomes, NGS provides comprehensive data on genetic variations (SNPs, InDels, and other mutations), allowing differentiation between strains that are nearly identical but have subtle genetic differences, which is a critical aspect of accurate source attribution [91].

In foodborne or waterborne outbreaks, mainly caused by *Salmonella* spp. (dairy products, meat, and vegetables), *Campylobacter* spp. (poultry), *Escherichia coli*, *Shigella* spp., and *Vibrio* spp. (seafood), and *Listeria monocytogenes* (prepackaged food) or its toxins (toxins produced by *Vibrio* spp., *Clostridium perfringens*, *Shigella* spp., *Escherichia coli*), NGS can be used to trace the origin of contamination by comparing genome sequences from bacteria identified from patients, food products, and environmental samples. By sequencing the genomes of microbes found in contaminated samples and comparing them to reference databases, investigators can identify the source, whether a particular food processing facility or a water supply system [92–94]. During the 2011 *E. coli* O104 outbreak in Europe, NGS was instrumental in tracing the outbreak to a specific batch of fenugreek seeds. This precision in source attribution aided a more efficient control of the epidemics and provided evidence applicable to legal and regulatory contexts [95].

4.4. Biodefense and National Security

Next-generation sequencing has profoundly impacted biodefense and national security by enhancing the capabilities of detection, identification, and response to biological threats. NGS can be employed to identify potential bioterrorism agents. Among the most of these agents are bacteria (*Bacillus cereus* biovar *anthracis*, *Bacillus anthracis*, *Brucella* spp., *Burkholderia mallei*, *Burkholderia pseudomallei*, *Corynebacterium diphtheria*, *Corynebacterium burnetii*, *Escherichia coli* 0157:H7, *Francisella tularensis*, *Rickettsia prowazekii*, *Rickettsia rickettsia*, *Salmonella* spp., *Vibrio cholera*, and *Yersinia pestis*) and its toxins (neurotoxins from *Clostridium botulinum*, Epsilon toxin from *Clostridium perfringens*, Stx1 and Stx2 from *Shigella*

dysenteriae, and T-2 toxin and tetrodotoxin from *Staphylococcus aureus*), virus (Encephalitis virus, Chapare, Crimean-Congo, Ebola, Guanarito, Kyasanur Forest disease, Lassa fever, Lujo, Machupo, Marburg, Omsk, Rift Valley fever, Sabia, Influenza, Avian Influenza H5N1, SARS, Hendra, Nipah, Human immunodeficiency, Foot-and-mouth, Monkeypox, Variola major, and Variola minor), and fungi (*Aspergillus fumigatus*, *Candida* spp., *Coccidiodes immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*) [2,96].

In biodefense, the time-efficient and accurate comprehensive genomic analysis rapidly determines the exact microorganism strain, its origin, and potential genetic modifications, which are critical aspects to distinguish naturally occurring pathogens from potentially engineered or weaponized ones. For instance, in the event of a suspected bioterrorism attack, NGS can be used to sequence the pathogen genome, providing information if it has been genetically altered to increase virulence, resistance to treatments, or transmissibility [97–99]. Moreover, by continuously sequencing pathogens from various matrices (e.g., environmental samples, clinical cases, and wildlife), this technology builds a detailed genetic database that improves the ability to monitor and track emerging biological threats. These databases can be used to detect unusual genetic changes or the appearance of novel pathogens, which may signal a potential bioterrorism event or a naturally emerging pandemic [75,100].

In the context of national security, NGS plays a vital role in attribution (determining the source of a biological agent, whether it is a state-sponsored attack, a terrorist group, or an accidental release), providing the genetic fingerprints necessary to trace the origins of a pathogen with high precision, frequently down to the specific laboratory or geographic region. This level of detail is pivotal in response to biological threats, as it allows for targeted and appropriate countermeasures, including diplomatic, military, or law enforcement actions [75]. Also, NGS allows distinguishing between intentional and accidental releases of biological agents. For instance, in the case of an outbreak, NGS can compare the genome of the pathogen with those in existing databases to determine whether the strain is a natural variant or one that has been previously modified in a laboratory setting. This information is crucial for understanding the nature of the threat and formulating an appropriate response [79]. Finally, detecting specific genetic mutations associated with resistance or virulence obtained using NGS-generated data helps more effective medical interventions against the identified threat. This capability is important in bioterrorism events where time is critical, and the ability to quickly develop and deploy countermeasures can save lives [73,84].

4.5. Forensic Genomics

In criminal investigations involving microorganisms, NGS can help establish links between samples found at crime scenes and suspects. For example, it can match strains of bacteria found in a suspect's possession with those found at the scene of a bioterrorism event or contamination incident [101,102].

4.5.1. Determining Cause of Death

Determining the cause of death (COD) is a cornerstone of forensic investigations since it provides insights to uncover foul play, validate witness statements, and guide investigations. It is essential for legal proceedings and public health, identifying potential threats, and informing preventive measures.

Post-mortem microbiology (PMM) has emerged as a valuable tool in forensic science, especially useful in cases where traditional methods of determining COD are inconclusive. The study of the types and abundance of bacteria, viruses, and fungi present in various tissues and fluids often elucidates the microbial factors that may have contributed to the death—detecting unexpected infections, confirming suspected infections, evaluating antimicrobial therapy efficacy, and even recognizing medical errors. Moreover, PMM is particularly useful in cases of immunocompromised individuals or on corpses in an advanced state of decomposition [103–106].

In this case, NGS provides a more accurate diagnosis of suspected but unproven infections associated with nosocomial infections (mainly caused by *Escherichia coli*, *Pseudomonas* spp., *Staphylococcus epidermidis*, rotavirus, adenovirus, *Aspergillus* spp., *Candida* spp.) [107] or sudden death cases (example: *Bordetella pertussis*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, adenovirus, cytomegalovirus, Epstein–Barr virus, human metapneumovirus, and respiratory syncytial virus) [108,109]. In both cases, the abnormal presence of a pathogenic virus or bacteria is analyzed, and the results can be confirmed with blood cultures, histological findings, procalcitonin and positive C-reactive protein (CRP) measurements, and antemortem clinical information [9].

Moreover, at least theoretically using rat models, NGS can be applied to discriminate between drowning and post-mortem submersion by identifying algae, diatoms (microscopic algae with a siliceous exoskeleton), and aquatic bacteria in the circulating blood or present in the internal organs of the victim [110,111].

Post-mortem dysbiosis (i.e., microbial imbalances in deceased individuals) has been reported to be associated with drug-related deaths, also providing insights into the cause of death. It has been suggested that the dispersion and composition of post-mortem microbiota could serve as a forensic tool for distinguishing between the manner of death (e.g., homicides vs. suicides) and the cause of death (e.g., cardiovascular disease vs. drug-related deaths) [112].

Another application of PMM is estimating the time since death (post-mortem interval—PMI), through the changes observed in microbial communities in and on the body observed after death (Section 4.5.2). This is particularly useful in cases where other methods (e.g., rigor mortis or body temperature) are less reliable.

Despite its utility, PMM presents several limitations, including small-scale studies, lack of standardized protocols, and limited access to human cadavers [3]. Therefore, PMM result interpretation requires careful consideration of clinical history, macroscopic findings, and histological appearances [103]. Also, a protocol should be established to ensure proper sampling techniques and contamination reduction [104]. Therefore, future research should address the identification of stable biomarkers within dominant bacterial phyla and develop machine-learning models to enhance PMM's reliability in forensic investigations [3]. Also, PMM's effectiveness should be improved by multidisciplinary collaboration among microbiologists, pathologists, and forensic physicians [104].

4.5.2. Estimating the Time since Death

Estimating PMI is a critical aspect of criminal investigations, especially in cases of suspicious deaths without witnesses. The accurate PMI estimation validates witness statements, enhances the credibility of additional evidence, offers reliable information for leads, streamlines resource allocation decisions, and serves as evidence for later legal proceedings. Like forensic entomology, the succession of microbes within different parts of the human body after death has been proven helpful in estimating the PMI, a critical point of any forensic investigation [9].

The human body harbors trillions of microorganisms. After death, the decomposing body becomes a nutrient source, attracting various microbes. The succession of these microorganisms at different degradation stages offers insights for precise PMI estimation. From an initial predominance of Proteobacteria, at later stages of decomposition, corpses become colonized by Bacteroidetes, Firmicutes, and Ascomycota fungi [113]. As such, NGS has been applied to follow the dynamics of the thanatomicrobiome (community of bacteria and fungi present in the blood and internal organs) and epinecrotic community (microorganisms and macroorganisms that interact with a decomposing body, influencing its decay process), acting as molecular clocks of decomposition [3,114–119].

Although PMM is promising in estimating PMI, several difficulties and critical issues arise from using it. The main challenge is associated with microbial communities' complexity and variability. After death, the human body undergoes significant microbial changes influenced by numerous factors (e.g., the deceased's health, environment, and the presence

of other organisms) that make it difficult to determine a universal timeline for microbial succession that applies to all cases. Also, environmental factors (e.g., temperature, humidity, and soil composition) further complicate PMI estimation., since they can drastically alter the rate at which microbial communities change post-mortem. Moreover, sampling and methodological issues are also significant. Microbial sample collection from a decomposing body can introduce contamination or miss key microbial indicators. Microbial genome instability adds another layer of complexity. As microorganisms adapt to the decomposing body and its changing environment, their genomes may mutate or undergo horizontal gene transfer, potentially leading to errors in interpreting PMI based on microbial data. Another challenge is the lack of comprehensive reference databases that catalog the microbial succession patterns across different environments, geographic locations, and conditions. Without them, the ability to accurately determine PMI remains limited. Finally, the use of microbial data in court requires robust validation and a clear understanding of the limitations of PMI estimation. Misinterpretation or overreliance on microbial evidence without considering its uncertainties could lead to wrongful conclusions in forensic investigations [120,121].

4.5.3. Determining the Crime Scene Location

Soil analysis, including mineralogy, chemistry, geophysics, texture, and color, is frequently used as evidence, establishing a link between a suspect and an object, location, or victim. With the advances in NGS technology in recent years, the soil microbiome has been regarded as valuable in forensics, providing discriminatory power at fine-scale resolution and allowing for the distinction of geologically similar samples from distinct environments [122–124]. Over the last years, several manuscripts published in microbial metagenomics have pointed out the existence of unique microbial signatures associated with different environments, highlighting the potential applications of metagenomic data in forensic science [125–128].

Soil microbial communities collected from shoe soles hold potential forensic value, as they can differentiate between soil samples from various locations, helping to establish links between suspects, victims, and crime scenes [129]. Soil analysis may resort to sequencing techniques of genomics such as 16S rRNA (bacteria and Archaea) or ribosomal intergenic spacer and 18S rRNA (Fungi) gene [130–133].

Another concern in forensic investigation is the distinction between primary (where the criminal act was committed) and secondary crime scenes (other places related to the event in a later time-lapse). Both scenes are critical for the reconstruction of crime. However, the primary scene frequently contributes more evidence to forensic research than the secondary crime scene. Still, unfortunately, in most cases, only the secondary crime scene is available for the investigators [8].

The forensic potential of the environmental metagenomic profiles was also recognized in the early stages of the MetaSUB International Consortium [134] and confirmed in a recent analysis of almost 6k samples collected worldwide during global City Sampling Days to build a whole metagenome sequencing-based molecular profile of cities [134]. In 2017, MetaSUB started collaborating with the Critical Assessment of Massive Data Analysis (CAMDA, www.camda.info) conference to develop, improve, and benchmark classification tools for metagenomic data in an open science-based contest. The potential of this approach was shown at the CAMDA conference in 2017, where a metagenomic classification challenge was posed based on MetaSUB data. Since then, the challenge has moved beyond constructing city-specific metagenomic profiles towards the applicability of predictive models in the following: (i) classification of a new sample from a known location (CAMDA 2018); and (ii) prediction of sample origin from an unknown location (CAMDA 2019, 2020). Interestingly, many applied approaches achieve a high classification accuracy [>90%]. However, results from the 2018, 2019, and 2020 challenges highlight the shortcomings in the original prediction of previously unknown samples. Analyzing the results and participants' feedback made it clear that a more extensive dataset [better geo-resolution] and richer meta information would benefit a proper assessment. On the other

hand, existing solutions might be good enough for some local/country-wise applications, but further investigations are required.

However, soil's nature is complex and heterogeneous and varies spatially and temporally, complicating microbiome analysis [135,136]. For instance, Singh et al. (2018) studied the changes in the spatial dynamics of human cadaver decomposition on soil bacterial communities at distances of 0, 1, and 5 m. These authors demonstrated that bacterial community composition at 0 m significantly differed from those at 1 and 5 m, with lower alpha diversity at 0 m due to the cadavers' nutrient input [137]. As for temporal variations, metagenomic studies of grave soil demonstrated the existence of seasonal fluctuations in microbial community succession, pointing out a possible interaction with environmental factors (e.g., humidity, heat exposure) [138].

4.5.4. Identifying Suspects

The human microbiome, based on stable autochthonous (i.e., native to a given environment) microbial profiles, constitutes a unique 'fingerprint' that can potentially distinguish between individuals and can thus be used for human identification purposes. This feature becomes particularly relevant when the recovered human DNA is not of sufficient quantity and quality to obtain a fully individualized DNA profile based on short tandem repeats (PCR-STRs), as when dealing with 'touched' samples linked to objects (e.g., mobile phones, computer equipment) [134,139].

The work by Franzosa and coworkers [140] demonstrated that body site-specific microbial profiles can identify individuals. For instance, gut microbiomes could identify nearly 80% of individuals up to a year later, although variability remains challenging. Moreover, other studies [141,142] have shown promising accuracy in identifying individuals through microbial analysis of various body sites. However, despite its potential forensic applications, the current methods lack sufficient accuracy, and improvements in sensitivity, specificity, and contamination control are needed, along with a deeper understanding of microbial dynamics over time and space.

Initially, it was thought that the skin microbiome—skin's surface microbial communities—could, at least theoretically, provide specific and individualized information, considered an asset in identifying and linking individuals to crime scenes [134]. Nevertheless, its accuracy in identifying individuals has decreased as the number of comparison individuals potentially sharing the same environment, lifestyle, and microbial patterns increases [143]. The skin microbiome is influenced by body microenvironment composition (anoxic microenvironment supports the growth of *Propionibacteria* spp. and *Staphylococcus* spp.; moist environments support *Corynebacterium* spp.; dry skin presents the highest diversity with variable populations and abundances of the phyla Actinobacteria, Proteobacteria, Firmicutes, and Bacteriodetes), being influenced by endogenous (examples: age and ethnicity) and exogenous factors (examples: diet and geography) [144]. The manuscript by Neckovic and collaborators revised the main constraints associated with individual identification based on the skin microbiome. Among the main limitations are microbiome transfer (i.e., indirect transfer event vs. direct contact) and persistence (i.e., naturally existing temporal shifts in community compositions), sample collection (despite the use of personal protective equipment (PPE) and the adherence to standard operating procedures (SOPs), human skin-associated bacterial communities can be transferred during a routine item examination) and storage (the storage conditions usually used for human DNA are not adequate for microbial DNA; for instance, cool and dry environments may induce changes in microbial growth and structure, producing misleading identification results), DNA extraction (i.e., depending on the extraction kit used, varying portions of microbial DNA can be extracted, harming the extraction reproducibility), and sources of contamination (e.g., bacteria such as *Propionibacterium acnes* and *Corynebacterium*, usual inhabitants of human skin microbiomes, are frequent contaminants of extraction kits) [145].

Hair is another common biological type of evidence recovered from crime scenes. However, most lack the anagenic root (actively growing hair) that allows human iden-

tification, such as the STR profile [146]. One of the most promising applications of the hair microbiome has been reported as the detection of the transfer of pubic hairs between individuals during sexual intercourse [147,148]. The pubic hair microbiota (*Corynebacterium* spp. in male samples; *Lactobacillus* spp. in female samples), although more stable and less affected by environmental bacteria than the scalp hair, can be influenced by factors such as geographical origin, intrinsic hair characteristics [144], and the frequency of sexual activity [146].

Where the body fluid microbiome is concerned, the differences found in microbial communities from different human body locations differ more than the microbial communities from a given body location among different individuals. The most commonly forensic body fluids include vaginal fluid, semen, saliva, and blood [143].

Identification of vaginal fluids is critical in forensic investigations, mainly in sexual assault casework. As previously reported, the dominant communities in healthy vaginal secretions are *Lactobacillus* spp., the microbial composition of vaginal samples considered relatively stable, irrespective of the female menstrual cycle stage or pregnancy. Nevertheless, medical conditions such as vaginosis can lead to dysbiosis, altering the "normal" vaginal microbiota. Research in this field suggests the potential to distinguish individuals using microbiota approaches in cases of sexual assault or violence [122,149,150].

The semen microbiome is mainly characterized by the presence of *Lactobacillus* spp. and *Staphylococcus* spp. At the same time, pathogenic bacteria can affect the seminal fluid's microbial composition, showcasing the potential of microbiota analysis in forensic studies, particularly in cases where spermatozoids are not observed [151,152].

Saliva is a common biological fluid encountered in crime scenes, originating from bite marks on victims from sexual assault or physical altercations, objects (e.g., cigarette butts, chewing gums, drinking containers, utensils, envelopes and seals), and spit (due to coughing, yelling, or other physical exertion). Saliva, containing nearly 500 million bacterial cells/mL and 700 bacterial species, presents a dynamic and variable oral microbiota. The stability of the oral microbiome over time, as well as its dynamics, has been explored, revealing specific individual stability that can persist over shorter periods but may vary over the long term, not being influenced by tooth brushing but influenced by antibiotic and probiotic consumption [153–156].

Blood is another frequent fluid found at crime scenes, originating from violent crimes (e.g., physical assaults, stabbings, shootings, or beatings), suicides, accidents with significative trauma (e.g., hit-and-run, car accidents, falls, industrial incidents), sexual assaults and domestic violence, burglary and home invasions associated with injuries and self-defense scenarios. Recent studies demonstrated that it is possible to distinguish various blood types (i.e., menstrual, venous, nasal, and skin epithelium) according to the microbiome's composition. Nevertheless, the taxonomic screening method requires further evaluation, reflecting microbiota-based forensic tools' ongoing exploration and refinement [157]. Distinguishing among these blood types is important in forensic investigations since it allows for identifying the blood source and understanding the crime context, linking evidence to individuals, detecting contamination, and ensuring accurate forensic analysis. This information provides critical details to reconstruct the events and validate evidence collected during investigations.

In broader forensic applications, studies investigating microbial communities in various samples, including mouth, vagina, feces, penis, and hand, have revealed site-specific species and microbial diversity. These investigations contribute to understanding the core microbiomes associated with different body fluids or sites, serving as essential preliminary results in forensic analyses. The evolving landscape of microbiota-based forensic tools underscores their potential to enhance the accuracy and reliability of forensic investigations across diverse scenarios [158].

5. Advantages of Next-Generation Sequencing Technology in Forensic Microbiology

As described in the previous sections, by providing a robust and high-resolution approach for analyzing microbial genetic information, NGS technology has revolutionized forensic microbiology, being applied to several fields, as the understanding of the dynamics of infectious diseases, the source of bioterrorism incidents, and resolution of civil and criminal cases.

Next-generation sequencing enables microorganism detection even at trace concentrations or in complex samples. This high sensitivity is particularly valuable in cases with trace amounts of microbial DNA (e.g., degraded, or mixed samples), allowing pathogens identification. Moreover, NGS can be applied to various sample types, collected using different methods and subjected to different treatments (e.g., formalin-fixed paraffin-embedded tissues, swabs, blood, and environmental samples), a versatility that is advantageous in forensic settings where the sample type may vary.

This technology identifies a broad spectrum of microorganisms (i.e., bacteria, viruses, and fungi) in a single sequencing run, providing a more realistic view of the microbial community and allowing the simultaneous detection of multiple pathogens that may interact during disease. Also, NGS allows distinguishing between closely related microbial strains, an important aspect in determining pathogens' source and transmission pathways in environmental contamination, disease outbreaks, or bioterrorism/biocrime attacks.

Compared to classical microbial methods, such as culture, NGS offers a faster turnaround time, making it possible to analyze multiple samples in a high-throughput manner, a critical aspect in forensic investigations, enabling faster and more informed decision making in response to potential public health threats.

Next-generation sequencing facilitates in-depth genomic characterization of microbial strains since it allows identifying and providing detailed information (e.g., genetic makeup, virulence factors, and antimicrobial resistance) on a specific pathogen. Such aspects offer invaluable insights into their potential impact, transmission patterns, and relatedness to other strains, facilitating more accurate epidemiological investigations.

Finally, NGS provides robust and evidence-based findings admissible in courts, strengthening forensic microbiology analyses' legal and scientific validity. The detailed genetic information obtained through NGS serves as evidence to confirm the presence of deliberate biological threats and link specific microorganisms to criminal activities.

6. Limitations of Next-Generation Sequencing Technology in Forensic Microbiology

Despite all its advantages, NGS technology also presents challenges and limitations which must be carefully considered when applying NGS to forensic microbiology. Next, information on some of these challenges is presented.

Genomic instability constitutes a significant challenge to a wider application of microbial forensics, leading to potential errors in identifying and characterizing bacteria and viruses. Microorganisms' genomes can undergo frequent genetic mutations, rearrangements, and recombination events that may make the reliability of forensic analyses difficult. In bacteria, genomic instability results from mutations in key genes or the acquisition of mobile genetic elements (e.g., plasmids and integrons) that introduce new genetic variations. These changes can alter the bacterial phenotype, affecting its identification and characterization. In forensic contexts, where precise strain identification is critical, such genomic variability can result in incorrect conclusions or misidentifications, particularly if the forensic analysis relies on specific genetic markers that may not be stable across all strains [159,160]. Viral genomes, especially those of RNA viruses, exhibit even higher levels of instability due to their high mutation rates and error-prone replication mechanisms, leading to significant genetic diversity within a single viral population. Variations in viral genome sequences may cause issues in aligning forensic data with reference sequences, leading to potential errors in determining the origin or characteristics of the viral pathogen [161–163]. Therefore, strategies to mitigate genomic instability must be imple-

mented to enhance the accuracy of data interpretation. These strategies may include using multiple genetic markers and incorporating robust bioinformatics approaches [164].

In individual identification, NGS presents a relatively long time-to-result when compared to other analytical techniques like standard PCR-STR. This conventional technique, a widely used method in forensic analysis, provides results much faster than NGS, with this more reduced turnaround essential in time-sensitive investigations. However, NGS offers a significant advantage that mitigates its longer processing time: the ability to sequence the entire genome in a single run, allowing the broad identification of genetics variants such as VNTR (Variable Number Tandem Repeats), STR, SNP (Single Nucleotide Polymorphism), and InDels (Insertions and Deletions). This comprehensive sequencing capacity allows for a more detailed and thorough analysis, revealing information that PCR-STR-PCR misses. By capturing the full spectrum of genetic variations, NGS provides in-depth information valuable in complex forensic cases, making its longer time-to-result a worthwhile trade-off in many scenarios.

In post-mortem samples, the quality of the collected biological material can be compromised due to decomposition, putrefaction, or other post-mortem changes. The degraded nucleic acid obtained from these suboptimal-quality samples can result in suboptimal sequencing results.

Biological samples collected from crime scenes or natural environments may contain environmental contaminants that interfere with accurately identifying the target microorganism. Also, NGS workflows are sensitive to laboratory contamination, and even small amounts of DNA from different sources affect the results' accuracy. Nucleic acid extraction remains the primary factor influencing the quality of data obtained using NGS. Another concern is the presence of mixed samples that require more complex wet-lab designs, including spike-ins.

The effectiveness of NGS in forensic microbiology relies on the availability of comprehensive and updated reference databases. Some microorganisms may still need to be characterized, making their identification challenging. In database design, there has long been an assumption that knowledge is limited to the observable and recordable. This perspective has made us aware of a tiny segment of global species [158]. The vast gaps in our scientific understanding have prompted a surge in the development of new ontologies, particularly for a deeper insight into the microbial universe. Functional ontologies, for instance, have already yielded substantial advancements in our understanding of microbial communities [165].

Next-generation sequencing generates massive data; bioinformatics analysis is highly complex and resource-intensive. Effective data management and analysis require specialized skills and robust computational resources. Developing and maintaining bioinformatics tools specialized for forensic microbiology analysis is also challenging, while creating standardized workflows and best practices is still an ongoing effort.

Interestingly, multiple approaches provide comparable results and could be of interest. In terms of metagenomic data processing based on CAMDA and MetaSUB work, we could divide these into three conceptual groups:

(a) Taxonomy/species-centric: in this "classical" approach, data are analyzed via taxonomy profiling to identify the presence and abundance of a given species;
(b) Functional approach: it has been shown that species composition is less important than the presence or absence of certain functions required by a microbial community to settle a specific ecological niche, which allows looking at the functional profile of a sample without prior taxonomic assignment;
(c) *K*-mer-based approach: as in microbiome studies, about half of the data cannot be assigned to any known species. It is beneficial to omit reference limitations and use all the data (from known and unknown species) directly to classify samples by their *k*-mer profile. However, due to feature space size, an efficient approach for relevant feature selection is needed.

As in any other forensic investigation, maintaining a proper chain of custody is essential to ensure the integrity and admissibility of the evidence during the trial. The potential for sample contamination at various stages must be carefully managed. On the other hand, the legal framework concerning the use of NGS data as evidence in court cases is still evolving, being necessary to convince legal authorities of the reliability and accuracy of NGS results.

While NGS quickly identifies genetic variations between distinct microbial strains, distinguishing between closely related strains remains challenging, requiring specialized expertise to interpret the significance of such variations. In epidemiological studies, differentiating between causative agents and bystander microorganisms can be difficult, mainly in co-infections.

NGS can be cost-prohibitive for smaller or underfunded forensic laboratories due to the cost associated with equipment and reagents acquisition or personnel training. Also, storing and managing large NGS datasets are resource-intensive and can become a logistical challenge.

Despite all efforts from scientists and law enforcement professionals, there is still extensive unavailability of standardized operational protocols (SOPs). As a result, inconsistent or inadequate sample collection and preservation techniques hinder the quality and usability of samples for NGS analysis in court. Ensuring that NGS technology meets forensic science's stringent validation and quality control requirements is an ongoing challenge.

7. Conclusions

The development of NGS methods has made it possible to study the human and environmental microbiome on a large scale. As a result, understanding the complexity and dynamics of microbial communities has paved the way for the development of new methods in forensic science. The practical importance of studying the microbiome is more than just about detecting harmful or dangerous pathogens of interest to forensic science. Microbiome analysis in forensic science can provide helpful information at the investigation stage and help clarify the circumstances of a criminal case.

Investigating the cause and time of death, body fluid identification, and DNA intelligence through detecting specific microbiome patterns in the environmental samples are just some of the applications of microbiome analysis in forensics.

The most used method of forensic microbiome analysis is 16S rRNA gene sequencing, but multi-target and whole-genome shotgun sequencing methods are becoming increasingly important. With the need for more precise and robust methods, the "outside of the box" solutions, often translated from the data science, are providing additional insight into the deeper levels of sequence processing, and they are incredibly reliable, even though some of those methods do not even use reference databases. The idea is to "talk to the data" and "let the data talk back", which means exploring the data, their properties, and metadata, and constructing the multi-paradigm experiment that will allow the use of all the information there is.

The *k*-mer-based approach, which can utilize all the reads, both coming from known and unknown species, should especially be more interesting for forensic applications as it will not depend on the evolving/updated references and stay consistent over time. It has shown its potential in MetaSUB work [126,166], but other approaches are still under investigation [167].

Future analyses should involve validation of the stability and accuracy of forensic microbiome analysis for its application in practice.

Artificial Intelligence (AI) models can potentially enhance data analysis, interpretation, and forensic accuracy. AI algorithms (i.e., machine learning and deep learning models) can handle the vast amounts of data generated by NGS technologies, identifying patterns, correlations, and anomalies that might be missed by traditional analytical methods, improving the sensitivity and specificity of microbial forensics [168–170]. Recently, AI-based language models, such as ChatGPT or Copilot, have revolutionized the process of data

acquisition and data analysis. These models utilize large amounts of text-based training data and a transformer architecture to generate human-like text in response to prompts. In the general context of forensic sciences, ChatGPT offers several advantages. These AI tools serve as virtual assistants, aiding lawyers, judges, and victims in managing and interpreting expert forensic data. Also, such models can assist in tasks such as data analysis, technical writing, and language translations, generating simple or advanced teaching materials. Its multilingual capabilities are not restricted to English, it processes prompts in different languages, and it even handles inputs like spreadsheets, research papers, and mathematical equations [171,172].

Nevertheless, large language models in forensic sciences raise ethical and legal concerns. While these models generate human-like responses, their accuracy and reliability need scrutiny. Relying solely on AI-generated expertise reports introduces errors or biases. It is critical to understand the underlying processes that allow an AI-based model to arrive at its conclusions. Transparency ensures that forensic experts, lawyers, and judges can assess the validity of its outputs. Finally, courts may question the admissibility of AI-generated reports, being necessary to ensure that these models adhere to legal standards [167,171].

ChatGPT-4, Bard, and Copilot are currently considered the most advanced publicly released AI models, with further improvements being developed. These models, along with other similar language models, can potentially shape forensic microbiology significantly. However, striking a balance between technological advancements and ethical considerations remains paramount.

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