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Review Article

# A Short Review of Forensic Microbiology

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## Abstract

**Background:** Microbial forensics is a multidisciplinary area, which has been recently considered an effective tool in forensic investigations. This growing field of forensics covers a wide spectrum of different branches of science, including biology, chemistry, physics, geology, mathematics, and computer sciences, leading to a practical approach that can be applied in several areas such as bioterrorist actions, environmental issues, emerging and reemerging diseases, as well as reliable trace evidence at a crime scene.

**Methods:** The information has been gathered via Google Scholar using several keywords, including forensic microbiology, bioterrorism, forensic investigation, and trace evidence. The data were from reliable articles and books published over 50 years. This paper is a short review of forensic microbiology with a bioinformatics perspective to use in different fields such as the court.

**Results:** It is known that using either microorganisms or their toxins is a low-cost potential tool with serious morbidity and mortality rates that can spread all around the world by food or water supplies or even through the air, making them a perfect candidate bioweapon with minimum traceability. Studies have indicated that environmental conditions plus biological and abiotic factors would affect the following analysis and the final validation, which is an essential step in the forensic investigation due to its highly effective role in the court vote. To face different challenges, law enforcement has the infrastructure for attribution and deterrence (e.g., following the exact microbial forensics program) so that it can be used in court. Developing more reproducible, sensitive, and accurate methods, preparing a wide reliable database, and devoting the right amount of budget will help improve the whole forensic procedure in the legal system.

**Conclusions:** The current paper is a short review of how forensic scientists can use microbial features on a crime scene to clarify and enhance the procedure to solve different criminal cases.

**Keywords:** Forensic microbiology, Bioterrorism, Forensic investigation, Trace evidence

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## Introduction

Microbial forensics is defined as the multidisciplinary major of science that uses scientific methods to analyze evidence in case of bioterrorist actions, or the inadvertent release of a pathogenic microorganism or toxin for determined ends (1).

This multidisciplinary major covers a wide variety of different sciences, including biology, chemistry, physics, geology, mathematics, and computer sciences (2).

It is a known fact that the use of either microorganisms or their toxin is a low-cost potential tool with serious morbidity and mortality that can spread worldwide by food or water supplies or even through the air, changing them into a perfect candidate bioweapon with minimum traceability.

Bioterrorism is an intentional use of biological agents, including microbes and their toxins mainly to cause terror and affect the economic situation (1).

As long as there is a risk of a bioterrorist action, forensic microbiologists should choose detection methods that are not only accurate to determine the exact agent but quick enough to prevent public panic in society, including DNA microarrays or biochips (3).

To encounter different challenges, law enforcement has the infrastructure for attribution and deterrence (e.g., following the exact microbial forensics program) so that it can be employed in the court (Figure 1). A successful microbial forensic investigation has special characteristics as follows:

- High sensitivity and specificity for detection and identification;
- Large rational databases;
- Validated analytical methods;
- Following quality assurance guidelines to obtain reliable results.

The present paper is a short review focusing on how

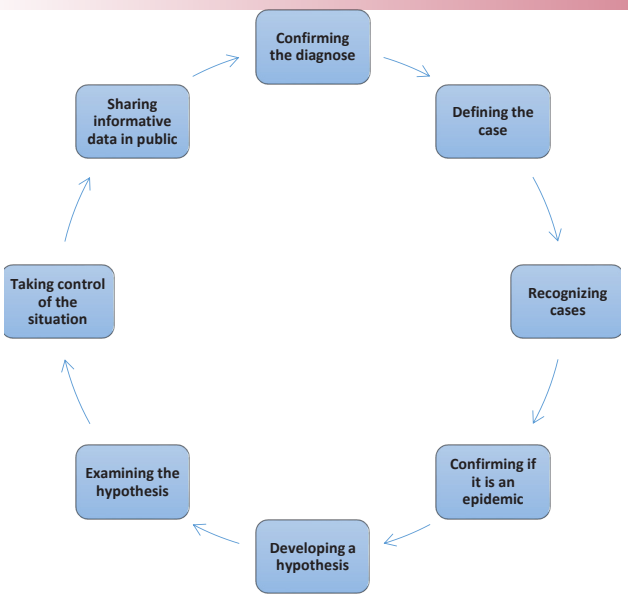



**Figure 1 .** Recognized Steps for Investigating Contagious diseases. Source. Centers for Disease Control and Prevention (11).

forensic scientists can apply microbial features on a crime scene to clarify and enhance the procedure to solve various criminal cases (1).

## History

Nearly two thousand years ago, the first bioterrorist activity was recorded, when ancient Romans deliberately poisoned their enemy's water source by bringing carrion into wells (4).

Although there were several signs of using biological agents in Homer's epic poems, Iliad and Odyssey, pointing out the fact of tipping arrows and spears with poison.

A few examples of bio crimes in history are the European plague epidemic during the Middle Ages (also known as Black Death), Smallpox and measles against Native Americans, Plague infected rats against the Japanese during World War II, and the International Shigellosis contamination in 1996 in Texas. In addition, the other examples include the anthrax letter in United States, anthrax cases among heroin users in Scotland, *Escherichia coli* O104:H4 outbreak in Germany, and the Ebola outbreak in 2014 (5-10).

One of the most common medical-related bio crimes involves dentists who infect their patients. In 1992, a group of scientists conducted research on the transmission of the human immunodeficiency virus in a dental practice. According to a phylogenetic analysis of HIV strains isolated from him and his patients, it was clarified that the mentioned dentist was in charge of infecting his patients during oral surgery (3).

Among all instances of microbial attacks in history, the anthrax letter of 2001 draw huge public attention, leading to the foundation of the Scientific Working Group on Microbial Genetics and Forensics on 29 July 2002 hosted by the Federal Bureau of Investigation (FBI) (1).

## Applications

As mentioned before, microbial forensics can be applied in various areas from a special medical condition to national security issues (e.g., in a case of a bio-attack). Solving environmental issues, investigating emerging diseases around the world, considering personal identification, determining the cause, time, and location of death, and examining the sexual assault cases are just a few examples of microbial forensics applications, which will be discussed in detail in the following sections.

### *Transmission of Disease: From Individual to Bioterrorist Action*

One of the forensic microbiology applications is perusing the source of disease whether they are endemic, epidemic, or pandemic. The important issue about the natural outbreak or bioterrorist action is the horizontal and vertical transmission, especially the rate of morbidity and mortality which can be divided into direct and indirect transmission categories.

The direct transmission includes direct contact such as touching, biting, sexual intercourse, or even animals and soils in case of zoonosis diseases, and direct projections are sneezing and coughs, leading to droplet spread.

On the other hand, indirect transmission can be listed as blood transfusion, physical or biological spread, and airborne transmission by dust.

To figure out a biological attack, there are some epidemiological clues published in 2003 as follows:

- When a disease is caused by an unusual agent;
- Emerging uncommon strains of agents;
- When a common sickness gets higher morbidity and mortality;
- Unusual distribution (either geographically or seasonal);
- An uncommon disease affecting a large population, especially for a specified age or group
- Similar genetic patterns between isolated agents;
- Enormous unexplained illness and death cases (12).

### *Deliberate Infection of Animals*

For over a thousand years, humans have been using animals to serve all sorts of their desires, including food, security, transportation, and the like.

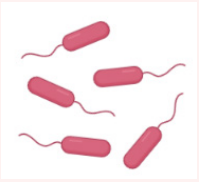
Animals were also involved in deliberate infection; according to ancient history, well poisoning has been documented as the earliest biological warfare. The rotting corpse of horses and rats was thrown down the water source, which was counted as the main reason for plague and tuberculosis transmissions. Nonetheless, some of the historical evidence seems to be remarkably affected by superstitious beliefs (13).

The anthrax letter has been considered the highlight of microbial forensics. Moreover, the role of Germany in the deliberate infection of animals during the world one epic is unavoidable. It has been reported that Dr. Anton Dilger produced a large batch of *Bacillus anthracis* and *Burkholderia mallei* agents which later caused infection in more than

3000 horses, cattle, and other animals that were supposed to be sent to European countries, including Romanian, France, and Argentina (14,15).

Rabbit calicivirus disease as a bioagent to reduce the rabbits' population in New Zealand (1997), the *Salmonella Typhimurium* contamination of salad bars in Dalles (1984), and emerging a water-born in Canada caused by *E. coli* O157:H7 and *Campylobacter jejuni* (2000) are also important cases related to the subject of illegally using animals to introduce a biological agent to specified population or location (16-18). Beside the mentioned cases, there are other microorganisms that had been applied as a bioweapon (Table 1).

**Table 1.** Few Identified Bacteria, Viruses, and Toxins That Could Be Used as a Bioweapon

| Bio Agents | Name | Disease |
|---|---|---|
| Bacteria  | *Bacillus anthracis* | Anthrax |
| | *Clostridium botulinum* | Botulism |
| | *Brucella species* | Brucellosis |
| | *Burkholderia mallei* | Glanders |
| | *Burkholderia pseudomallei* | Melioidosis |
| | *Coxiella burnetii* | Q fever |
| | *Escherichia coli* O157:H7 | Hemolytic uremic syndrome |
| | *Francisella tularensis* | Tularaemia |
| | *Salmonella* species | Salmonellosis |
| | *Salmonella typhi* | Typhoid fever |
| | *Shigella* species | Shigellosis |
| | *Vibrio cholera* | Cholera |
| | *Yersinia pestis* | Plague |
| Viruses  | *Arenaviruses* | Junin and Lassa fever |
| | *Ebola* virus | Ebola virus haemorrhagic fever |
| | *Lassa* virus | Lassa fever |
| | *Marburg* virus | Marburg virus haemorrhagic fever |
| | *Variola* major | Smallpox |
| Toxins  | Botulinum toxin | Botulism |
| | Ricin toxin from Ricinus communis | No explanation |

Source. Hawksworth and Wiltshire (19).

## Trace Evidence

There are enormous applications of different branches of microbiology, especially mycology in forensic sciences, including considering post-mortem intervals, determining the cause of death, locating corps, and the like.

One of the most valuable documents, using fungi and their spores, as well as bacterial spores, is tracing evidence, which has been proved to be useful in court (20).

## Microbial Decomposition

During the decomposition process, microbial communities will change and affect not only the corpse but also its surrounding environment. It should be noticed that any changes during the time would remain as trace evidence and could be different in each ecosystem.

Studies demonstrated that there is a microbial decomposer pattern, including bacteria, fungi, and non-fungal eukaryotes during decomposition (Figure 2). When the corpse is still fresh, the Proteobacteria is the dominant species but as time goes on, Bacteroidetes and Firmicutes will take the place, as well as Ascomycota fungi (21).

Examining the microbial ecosystem using soil or dust properties around the crime scene, bacterial and fungal spores, as well as plant substances could also be a highly useful forensic tool for predicting the exact geographical location and even the time of a specific incident.

Following standard collection and preservation of this evidence, different microbial DNA analyses would clarify the whole prokaryotic and eukaryotic community (23-25).

## Determining the Time of Death

Postmortem proliferation of the internal microbiome can be used to estimate the time of death, as well as to identify the possible victims and suspects involved in a crime. It should be noted that various factors from temperature and humidity degree to age and gender have different effects on corps even at the very same time and place (26-28).

While Forensic pathologists use core body temperature to estimate post-mortem interval, microbial forensics has been proved to be a useful tool for determining the time of death.

In 2009, a group of forensic scientists faced a crime scene in a closed apartment, where a man's body was stabbed; they found fungal colonies on carpets and couches. Therefore, they recreated the crime scene by adding those fungi to a mixture of a clean part of the carpet and bovine blood and then compared the results to the original fungal growth back there, which led them to realize that the death had occurred 5 days before finding the corpus (29).

## Postmortem Bacterial Translocation

There are several immunological and physical barriers in the human body to prevent bacteria from entering the other parts of the body.

After death, as the intestinal barrier disappears, bacteria enter the bloodstream (to name a few, *Bacteroidetes*, *Enterobacteriaceae*, and *Staphylococcaceae*) and different organs, including the liver, pericardial fluid, and spleen, which are important for bacteriological mortality after death. Bacteria can be detected by cultural methods or real-time quantitative polymerase chain reaction (RT-qPCR).

Blood is the first place where bacteria can be found soon after death. The pericardial fluid remains the most sterile, and the liver remains sterile for up to 5 days after death.

An autopsy can detect bacterial infections after death, including respiratory, administrative, and abdominal infections.

Additionally, post-mortem bacteriology could provide useful data on infection-related mortality when the

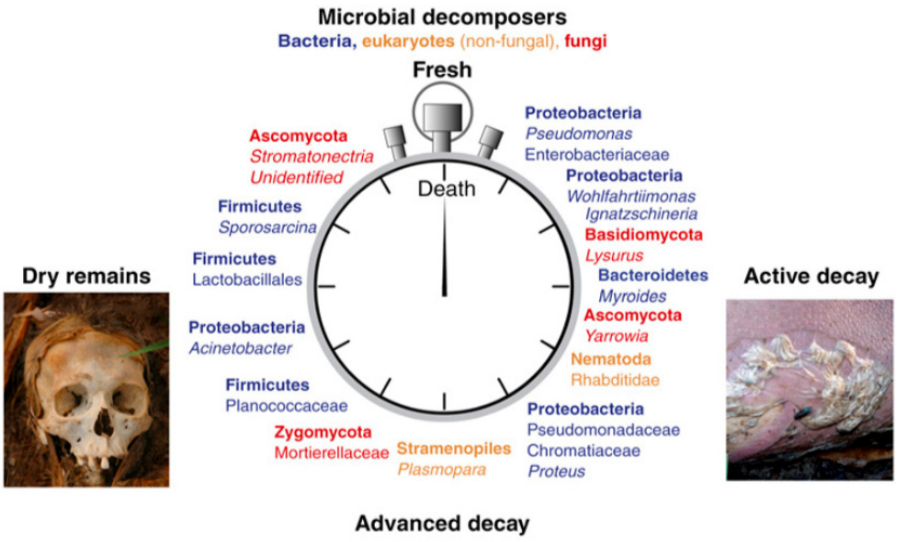



**Figure 2.** Microbial Decomposer Clock. *Source*. Allwood et al (22).

patient's history is unavailable (30,31).

### *Determining the Cause of Death*

There are three main problems for isolating and detecting infectious agents, including post-mortem movement, losing vulnerable species, and over-growth of faster-growing organisms. Different microbiological tests can be employed to determine whether a victim is drowned.

Diatoms are useful organisms for classifying unknown drowning victims since they might remain in the liver, bone marrow, and kidneys and could be detected by microscopic examinations and PCR assays.

Examining fecal bacteria such as *E. coli* and *Enterococcus faecalis*, which are mostly present in freshwater, is another sensitive tool for identifying drowning cases. Bacteria are present in the drowning medium, thus they can enter different tissues of the body and could be simply detected by growth in special culture media (32).

Bacterioplankton is also used as a marker for assessing drowning. Some studies demonstrated that bacterioplankton capable of proliferating in human blood not only reveals the salinity of water but also prevents bacterial contamination in blood (33,34).

### *Human Identification*

Similar to genetic codes and fingerprints, the human microbiome is also unique. Therefore, it can be applied for serving forensics and security service purposes. In addition, the specific microbial signature that can be found on hair, skin, body fluids, respiratory tract, intestine, genitals, and even feces plays an essential role in developing the personal medicine as each person would receive their personal medications(Figure 3).

#### *Human Skin*

Human skin is covered with various types of microorganisms, which are remarkably unique to each person, and as a result, this characteristic could be used to identify victims or criminals by tracing the microbial DNA (35). Moreover, it should be noted that in the case of sexual assault, a group of professionals must gather together to inspect all aspects of the case (Figure 4).

It is noticeable that when an object hit the body, not only does it burses the skin but also its effects on the microbial community will remain for a while.

This specific sort of evidence is particularly valuable in the case of sexual assaults or any other physical violence, including domestic violence such as child abuse in all sorts of ways (36,37).

#### *Human Hair*

Hair is another piece of biological evidence in forensic investigation. Microbial communities can be detected by means of human DNA profiling tests or even special cultural methods.

In line with the mentioned issue, high-resolution optical microscopy, electron microscopy, and scanning electron microscopy or nuclear DNA and mitochondrial DNA tests can be used to investigate the nature of damaged hair fibers. Variations in the severity of hair keratin loss can be considered for identification (38,39).

In addition to microscopic and molecular techniques, other characteristics such as even slight differences between hair colors showing decreasing the pigment could be the sign of probable sunlight exposure (40).

#### *Human Body Fluid*

Considering that each person has a specific microbial composition of body fluids, especially semen and saliva, these fluids could be used as detectable evidence at a crime scene which later can be examined by means of the next-generation sequencing technique.

It is a known fact that the vitreous fluid is more resistant to microbial decomposition compared to blood, saliva, or even semen, which makes it suitable to be examined for postmortem ethanol levels (37,41,42).

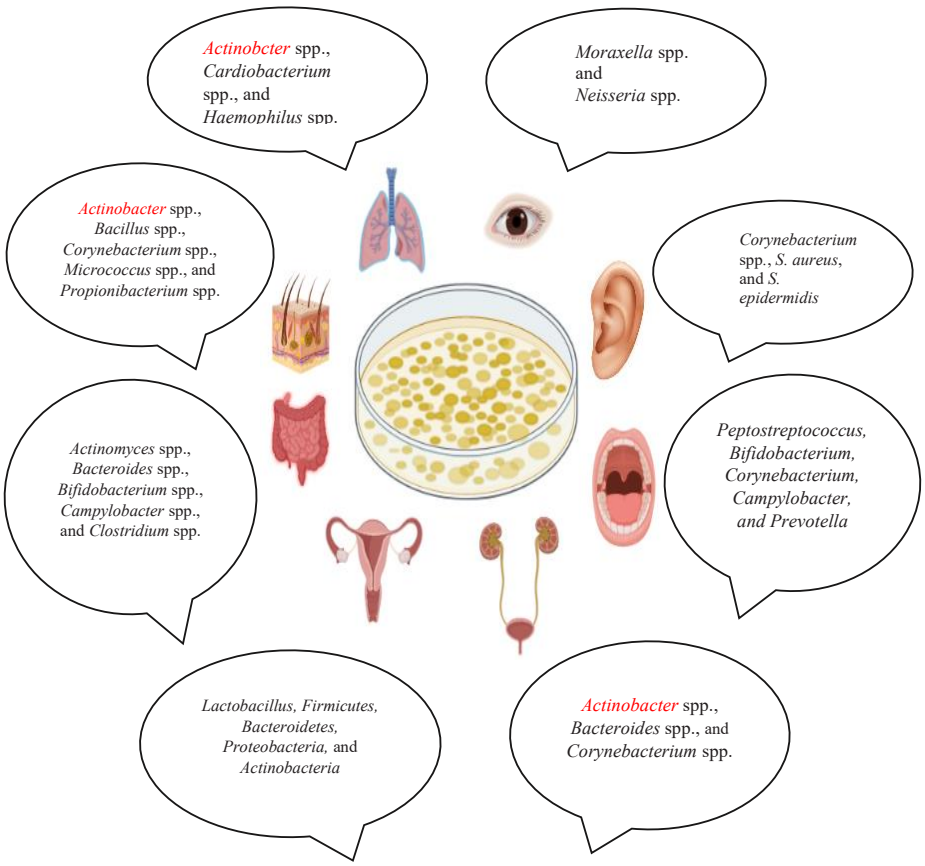



**Figure 3 .** Human Microbiome Can be Used as Unique Signature to as a Detectable Evidence in Forensic Investigation.

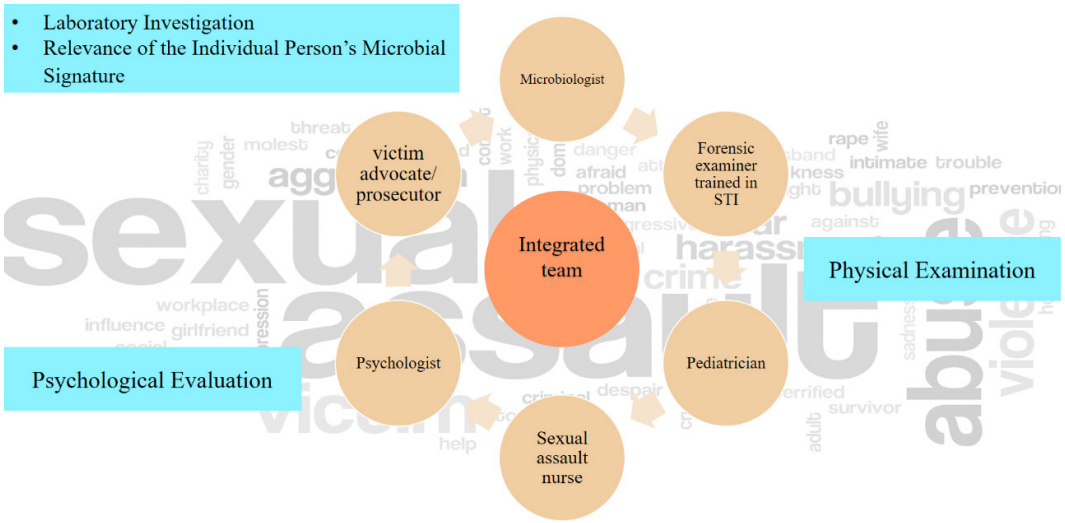



**Figure 4.** In a Case of Sexual Assault, a Professional Team Gather Together to Examine and Help the Victim and Find the Possible Offender .

Based on previous evidence, alcohol consumption is the main contributing reason to traffic accidents, drownings, suicides, and the like. When blood samples are unavailable, vitreous fluid can be occasionally examined as an alternative (43).

## Tools

In line with forensic investigation, all sorts of evidence play a role in collecting information, including the people, place, laboratory equipment, forensic tests, and even the time of the crime.

Since genome analysis could help diverge a bioterrorist action and a natural outbreak, DNA typing methods are the main tools for the attribution of pathogens.

Although DNA extraction methods need to improve to obtain more accurate results, After DNA extraction, molecular assays such as sequencing, microarray, pathogenicity array, single-nucleotide polymorphism, 16S rRNA sequencing, antibiotic resistance, and other methods will be performed to achieve the suitable

pathogen resolution.

In addition to mentioned DNA-based methods, there are other microbial assays to participate in solving a crime, some of which are presented as follows:

- Checking the morphology of microorganisms and trying physiological approaches such as phage typing, serotyping, and the like;
- Conducting isotope analysis to figure out the age and the origin;
- Examining the left growth medium;
- Detecting specific local bacteria;
- Using immunoassays to observe immunological responses.

In the case of using microbial toxins, immunoassays and mass spectrometry alongside DNA- based approaches would help trace evidence and find the origin of the toxin (1,44).

## Molecular Typing

During the time that the whole world is dealing with an outbreak, it is vital to detect the infectious agent and its therapy. Therefore, phenotypic and genotypic methods can be employed for identifying and differentiating strains.

### *Phenotyping Tests*

Reproducibility, ease of performance and interpretation, and ability to type all strains are three important characteristics that should be noticed for choosing phenotyping tests. Biotyping methods and antimicrobial susceptibility patterns are easy to perform and can type all strains. Multilocus enzyme electrophoresis is a phenotypic method, which not only has two earlier options but also is an excellent reproducible test. On the other hand, serotyping and phage typing are somehow difficult methods to perform and are not common among all strains (45).

### *Genotyping Tests*

Another kind of microbial test helping the forensic investigations is genotyping methods, which can be listed in three parts as follows:

1. Restriction endonuclease-based methods, including RFLP with and without hybridization;
2. Amplification-based methods such as RAPD, AFLP, and VNTR;
3. Sequence-based methods, including multilocus sequencing typing (MLST), electrophoresis karyotyping, single nucleotide polymorphism (SNP) analysis, and last but not least whole-genome sequencing (46-48).

### *Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)*

MALDI-TOF MS is a high-performance technology focused on a comparison of the protein fingerprint produced by microbial cells with the reference spectrum database using various algorithms on the species level, which is applied in various clinical identification occasions for both bacteria and fungi. Using this method in forensic investigation has also some challenges, including a vast reliable database and the ability to differentiate related biothreat agents (49,50).

Proteomics approaches can occasionally provide important data that cannot be achieved from DNA-based methods such as MALDI-TOF MS and LC-MS. Although metagenomics has recently become the standard method for identification and classification, it can be used in the criminal justice system (51).

### *Massively Parallel Sequencing Approach*

Metagenomic analysis has mainly concentrated on the 16S rRNA gene, which is inadequate for distinguishing subspecies. In addition, using a single gene target leads to other restrictions, including copy number variation, sequence variability among a single bacterium, and horizontal gene transfer. Therefore, scientists need to increase sampling and extend databases while adding new genomic sequences to advance specificity. To this end, highly developed technology and costly validation are necessary (52-55).

Using multiple phylogenetic markers, especially housekeeping genes (containing conserved and variable regions) has proved to be a suitable way to obtain more information for species resolution (56).

MLST is one of the methods, with the aid of bioinformatics tools and the use of 20 genes, for identifying bacteria (57).

Considering the limitations of these methods, there are other approaches to conduct, including multilocus variable-number tandem-repeat analysis, SNP analysis, and ribosomal MLST (58).

## Bioinformatics

Given the growing use of microbial forensic tests in the criminal justice system, optimizing computational approaches for downstream applications is an important challenge, resulting in overcoming bioinformatics. For instance, studies indicated that among different bioinformatics pipelines, including MG-RAST, Mothur, and QIIME2, the last one would be the suitable method for large postmortem microbial communities (59).

The findings of a study demonstrated that using hidSkinPlex as a new enrichment method could help forensic scientists profile the human skin microbiome and as a result, human identification (60).

## Validation

Validation is a necessary step in microbial forensics since not only the procedure but also the results can highly affect public health, political decision, and court vote. This process is summarized as follows:

1. Assessing the ability of procedures to obtain reliable results under defined conditions;
2. Rigorously defining the conditions that are required

to obtain the results;
3. Determining the limitations of the procedures;
4. Identifying the aspects of the analysis that must be monitored and controlled;
5. Forming the basis for the development of interpretation guidelines to convey the significance of the findings (61).

As stated earlier, microbial forensic findings could be the actual evidence before the law; therefore, the whole procedure should follow a valid paradigm from the moment the forensic team enters the crime scene or face a bio-attack until the moment that the results are submitted to the court.

Moreover, when it comes to bioterrorist attacks, so many governments might take responsibility. It takes at least four sovereign nations to join the international investigation and each will be subject to one critical task as mentioned below (61).

- Taking military actions;
- Taking internal police actions;
- Taking internal regulatory actions;
- Supporting political actions.

## Conclusions

As mentioned before, DNA extraction is an essential part of microbial forensics examinations. Thus, novel proper extraction and sequencing methods to develop reproducibility, sensitivity, and accuracy are crucial targets. In addition, finding a relation between post-mortem gene expression and microbial colonization would be a new area of interest.

Ultimately, almost every identification approaches rely on established databases, which needs to be expanded not only for human microbiome signatures but also for illustration of local microbial patterns in different areas, pollution source tracking, geolocation, Post-mortem interval (PMI) , and the like.

Some countries only use microbial forensic examination in special situations like sexual transmitted disease (STD) victims. Considering the widespread applications of this scientific field in solving crimes, especially bio-crimes, different legal systems could use its advantages to establish a proper chain of custody and obtain more accurate results.

### Conflict of Interests

The authors declared no conflict of interests related to this paper.

### Ethical Approval

Not applicable.

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