Chapter 17
Microbial Forensics: Beyond a Fascination

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Abstract Microbiology has seen a great transition from culture-based identification of microbes using various biochemical and microscopic observations to identify and functionally characterize the microbes by just collecting the DNA and sequencing it. This advancement has not only moved in and around microbiology but has found its applications in fields which were earlier considered to be the remote ones. Forensics is one such field, where tracing the leftover evidence on a crime scene can lead to the identification and prosecution of the culprit. When left-over microbes in the biological material or objects used by the culprit or the person in question are used to correlate the identity of the individual, it takes us to the new field of science—“microbial forensics.” Technological advances in the field of forensics, molecular biology, and microbiology have all helped to refine the techniques of collecting and processing of the samples for microbiological identification using DNA-based methods followed by its inference in the form of evidence. Studies have supported the assumption that skin or surface microflora of an individual is somewhat related with the microflora found on the objects used by that individual and efforts are ongoing to see if this is found consistently in various surroundings and with different individuals. Once established, this technique would facilitate accurate identification and differentiation of an individual or suspect to guide investigations along with conventional evidence. Legal investigations are not only the field where microbial forensic could help. Agriculture, defense, public health, tourism, etc. are the fields wherein microbial forensics with different names based on the fields are helping out and have potential to further support other fields.

Keywords Microbial forensics · Next-generation sequencing · Metagenomics · Microbiome · Culture-independent techniques · DNA

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17.1 Introduction

Tracing the leftover evidences on a crime scene remained the only way of getting to the culprit. The history and evolution of forensic sciences has been very fascinating and interesting for even those who do not understand the science behind it. The journey started with William James Herschel sometimes in 1858 when he recognized that the fingerprints remain unique to the individuals. It was used on legal and administrative documents then and was published in 1916 with all evidences and their analysis [1]. It was gradually picked up for all possible uses including crime scene investigations. Fingerprints remained the landmark evidence in all forensic investigations and are still playing important roles. Additional techniques came in to support forensics in the form of DNA fingerprinting, wherein DNA matching became useful in various cases. DNA fingerprinting is used to establish a link between biological evidence and a suspect in a criminal investigation [2–6]. DNA—or genetic—fingerprinting relies heavily on the principle that no two individuals share the same genetic code.

Recently, there has been a new discipline co-emerged with culture-independent techniques of identifying microbes. This new discipline looks for microbes and tries to co-relate them with individuals as like fingerprints or DNA. There is a constant interaction of individuals with microbes in their surrounding, and they leave microbes into their surroundings. Whenever there is a physical contact, bacteria hop across from the skin to the material used. The microbial communities attached with an individual's skin or other sites are being explored, and preliminary evidences suggest that they are unique and can identify individuals or the material used by them in a few cases [7–9]. This became the base for coining of a new term "microbial forensics." However the field has other dimensions too and would be discussed in detail in this chapter. The microbial forensics rely on the inputs from various fields of basic and applied sciences. These include microbiology, genetics, bioinformatics, forensic science, immunology, population genetics, biochemistry, molecular biology, epidemiology, etc. along with the law enforcement, public health, policy, and intelligence communities.

17.2 What It Entails

Bacterial density on the human skin may be as high as 107 cells/cm^2 [10] and can be freely transferred to surface which comes in contact. Evaluating the traces of skin microbiome left on questioned objects may be useful for forensic identification. Calculating the distances from samples and their donators, it seems possible to estimate whether items or palm prints belong to one specific person or not. This has become possible because of the culture-independent method of tracing all microbial species present in a given environment. This technique used here is called next-generation sequencing, and the population of microbes no matter cultivable or non-culturable is called microbiome. In this process, total DNA of the given environment or object is taken out, and the specific gene for prokaryotes, i.e., 16SrRNA gene, is amplified. This gene has a property like a clock with fast-moving arm and slow-moving arm. This concept has been explained by the work of Karl Woose who named this gene as a biological clock [11–14]. The gene contains highly conserved regions to identify the microbes with consistency and at the same time some variable regions to differentiate between closely related microbes. The technique has advantages over DNA from individuals as the microbiome DNA is abundant in touch DNA, and these organisms are much more stable because of complicated cell wall structures. Hence, it might be easier in certain instances to extract bacterial DNA than human DNA from surfaces target samples. Another good reason for this is the higher abundance of bacterial cells on the skin and shed epidermal cells as compared to human cells. There are some surfaces like fabrics, smudged surfaces, or highly textured surfaces from where obtaining clear fingerprints is very difficult. Bacterial DNA can still be found there and can help in solving the purpose [7].

17.3 Technical Terms and Their Explanation

Microbiology and forensic science were always considered to be different fields, and establishing a link between the two for better investigative power was not thought of earlier. Microbiology is simply defined as study of microorganisms wherein microorganisms are the organisms that exist as single cells or cell clusters and must be viewed individually with the aid of a microscope. Microbiology classifies microbes into various groups, and based on their characteristics and physiology, they could be assigned different genera and species. Most of the microbes, especially the saprophytic ones, depend on other substrates and interact specifically with their environment and habitat to gain important nutrients for their survival. This requirement remains very specific and unique with respect to one community of microbes and here comes a link with forensic sciences. Forensic science explores about those unique things (belongings or body remains) which could be co-related with the career (criminal) of those things, for instance, fingerprints or DNA. Because microbial communities on a particular individual's body are expected to be unique, identifying that individual with leftover microbial traces on the material used by the individual is a possibility and is being confirmed using various experiments. However, culturing of all those microbes and identifying them is a near impossible task. The reason is that all microbes do not grow on known culture media, and some which can grow are sometimes overtaken by the competition with other microbes in the same community. Hence collecting the total DNA and amplifying for the microbial signature gene is a good solution (Fig. 17.1). This technique is called metagenomics. Further, a sequencing technique that sequences the metagenome is called next-generation sequencing (NGS). NGS also known as high-throughput sequencing, represents different modern sequencing technologies for sequencing DNA and RNA much faster than Sanger sequencing and with lower economical and technical inputs.

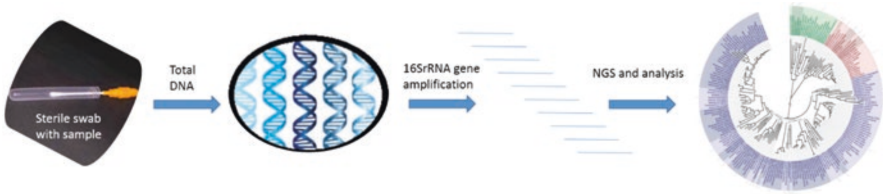

Fig. 17.1 The work flow of metagenomics from sample collected by swabbing a surface

17.4 How Does It Go Along with Other Techniques?

Identifying individuals is important in forensic science, and various developments have supported it from time to time. However, while making use of DNA, its quantity becomes crucial for accurate detection with desired quality for prosecution of crime. A smart offender can be cautious enough to decrease or degrade the traces of biological leftovers like blood, semen, etc. along with the fingerprints from the crime scene, which can complicate offender detection. Hence although fingerprints and DNA fingerprints remain very precise techniques, the availability of raw material becomes limiting factor in their use. Microbes can help in such scenario as their DNA is not as easily destroyed as a human DNA and remains available on surfaces at crime scene or on objects used. The pattern of bacterial DNA is dictated by the surrounding environment and the individual's microbiome [15, 16]. It is possible that the different bacterial patterns or the type of bacteria with typical physiology could discriminate individuals with different lifestyles. Hence, bacterial DNA analysis may serve as a complimentary technique in cases where standard DNA identification is partially informative [17]. Along with this, technologies like PCR, real-time PCR, MLST (multilocus sequence typing), MLVA (multilocus VNTR analysis), FISH (fluorescence in situ hybridization), and microarrays are being employed based on the need of the case or the availability of the infrastructure. Additional new methodologies like matrix-assisted laser desorption/ionization-time of flight (MALDITOF), gas chromatography-mass spectroscopy (GC-MS), and liquid chromatography-mass spectroscopy (LC-MS) are also well established in resolving minor difference in bacterial strains.

17.5 Scope of its Use

Investigation of a crime scene is not just potential application wherein microbial forensics can play an important role. Bioterrorism, biosecurity, biometry, medical forensics, etc. are the upcoming fields wherein definitive detection of microbes and their correlations may help a lot.

17.5.1 Criminology

Research on the transmission of microbes between human and surrounding environments has proved to be a property with potential for microbiome to be used in forensic investigations. In some cases, typical human microbial associations have been used to relate individuals to objects they have used [7]. Pattern of associated microbes with the surfaces at home and the family living and working with them have shown a predictive correlation to an extent that family's home and that individuals within a home can be differentiated [18]. Similar is the case with smartphones which rather remain in contact with specific individual and for longer durations [19]. Interestingly the microbial communities were different on the top and bottom of the phone differentiating the individuals and surface flora. An interesting property of the deposited microbiome is that it changes with a constant rate on a particular surface and can be utilized for forensic calculations. Work by Metcalf and co-workers have revealed that postmortem, the microbiome of animal hosts changes radically, but the pattern is much more predictable [20]. This can help tracing the time and direction of the events. Lax and co-workers worked to determine if the surface type and individuals have an effect on the microbial communities and have found that microbial community structure was determined both by surface type and participant.

Another aspect of microbial forensics is its role in bioterrorism. Herein the microbial forensics could be defined as "the discipline of applying scientific methods to the analysis of evidence related to bioterrorism, biocrimes, hoaxes, or the accidental release of a biological agent or toxin for attribution purposes" [21]. Microbial forensics, while dealing with bioterrorism, concentrate on identification of the agent or toxin and/or the mode of its production and dissemination. In addition, traditional forensic methods are used in conjunction to reach the goal of identifying the perpetrators of the crime. Around 14,000 microbial species or strains are listed as dangerous for humans [22]. Building individual diagnostic methods for these many numbers of agents is an impossible task, and hence NGS is the only tool to do massive parallel sequencing and to identify unknown pathogens, microorganisms modified to create panic, and pathogens in complex communities or samples with low abundance.

17.5.2 Agriculture and Medicine

With the global expansion of trade and communication, frequent movement of individuals from one country to other is unavoidable. In such scenario carriage of an endemic pathogen or drug resistance to a new geographical region is a looming threat. Recent outbreaks and transmission incidents of SARS and EBOLA have raised an alert. Microbial forensic has a crucial role in such cases wherein the status is not declared by individuals, is not detected by routine quarantine, or is a new pathogen altogether. Detection of microbial drug resistance or the emerging resistance is becoming increasingly important for human health. Similar things are true in the case of plant pathogens and food borne diseases [23, 24].

The foodborne diseases have always been a substantial global challenge to public health. A huge population worldwide become sick of foodborne illnesses every year with a substantial burden on public health as well as on economy. Addressing this problem has many steps, out of which one age-old problem is the rapid identification of the food source of the contamination. In a classic laboratory study, we could trace back the source of the foodborne outbreak, but the finding could not be utilized in helping the troubled ones immediately [25]. This is due to the infrastructural limitations and technical challenges in identifying the pathogens. A technology which was considered to be reliable and was used till recent past was pulsed-field gel electrophoresis (PFGE). However its resolution in pinpointing the source of the outbreak has not been satisfactory. Recent employment of whole-genome sequencing (WGS) in such investigations has shown promise. A retrospective study by US Food and Drug Administration's Center for Food Safety and Applied Nutrition (FDA-CFSAN) in 2012 could provide a far better resolution of the causal factors. All the isolates were sequenced on the Illumina MiSeq. WGS using Illumina could distinguish all of the isolates which looked exactly the same. Ultimately they concluded that the isolates from the outbreak were most closely related to a 5-year-old historical isolate that was linked to a processing facility only 8 km away from the source of the outbreak [26]. This could not only allow newer findings but also traced back the source of contamination to further allow the rectification. Such cases are sometimes accidental but are often criminal too, and tracing back the source would prevent such cases. In bio crimes, serious disease outbreak by natural occurrence or intentional may result in harm or death, causing disruption, creating fear, and affecting economic well-being. Microbial forensics thus plays an important role in consumer protection, food security, and even in litigation.

Agriculture and agricultural goods are also the susceptible area for microbial interventions and hence are important in terms of microbial forensics. There could be deliberate misuse of microbes or their products affecting flora and fauna which is important for agriculture. This could be given a name such as "agroterrorism" [27]. A field of investigation emerged against this threat for investigating into the violations and used scientific knowledge and technology to do so. This was given the name of bioforensics [28].

Another aspect of microbial forensic application to the foodborne pathogens is to trace pathogens in cash crops especially spices and other costly ones. Van Doren and co-workers have studied 14 reported illness outbreaks from Canada, Denmark, England and Wales, France, Germany, New Zealand, Norway, Serbia, and the United States which occurred due to consumption of pathogen-contaminated spice during 1973–2010. The outbreaks were reported from a few developed countries only. The reason for not including other countries was that those countries did not have updated technology to investigate and report similar findings. It was reported that these outbreaks resulted in 1946 human illnesses, 128 hospitalizations, and 2 deaths. Infants/children were the primary population segments impacted by 36% (5/14) of spice-attributed outbreaks [29]. The economic aspect associated here is the detection of pathogens after shipment and then the recall of the material. This involved a huge cost.

Recent development in microbial forensics in agricultural sciences also aids in pest control as well as deliberate introduction of pests along with food imports or use of pathogens as anticrop bioweapons. Different companies are coming up with molecular detection tools for rapid detection of specific pathogens in such products. For instance, Hu and co-workers have compared and evaluated the effectiveness of the molecular methods (3M Molecular Detection System (MDS) and ANSR Pathogen Detection System (PDS)) for the detection of *Salmonella* in egg products and compared the same with culture methods to find that the molecular methods are the superior and faster ones [30]. Budowle and co-workers have established a criterion comprising a foundation for investigators to establish, validate, and implement high-throughput sequencing (HTS) as a tool in microbial forensics [31]. Likewise, design principles for an effective microbial forensics program for law enforcement and national security purposes have been provided [32].

Microbial pathogens or toxins can be used to commit acts of terror; they can be used as weapons for execution of a crime. In biological warfare, transmissible lethal agents are used to attack the targeted populations. The impact of the bioterrorism was seriously considered after the anthrax attack in the United States in 2001. This incidence in the United States helped the world understand that bioterrorism can have drastic and global impacts. Microbial forensics has a role in such cases by applying scientific methods for the analysis of evidence from such a bioterrorism attack. Microbial forensic in conjunction with epidemiology can try to decipher if an outbreak is natural, accidental, or intentional [33, 34]. For instance, study by Price and co-workers found that the *Bacillus anthracis* injectional anthrax cases were originated from heroin users in Scotland [35]. Ou and co-workers used molecular tracking of HIV to report for the first time about the passage of HIV infection from dentist to patient after invasive healthcare procedure [36]. A Spanish anesthetist infected 275 patients with hepatitis C virus which could be found using phylogenetic and molecular clock analysis [37]. In the 2014 Ebola outbreak, the origin and transmission could be traced using bioforensic methods [38], etc. Other important and relatively new aspect of microbial forensics in medicine and medicolegal field is "thanatomicrobiome" (*thanatos*—death) that studies the microorganisms found in internal organs and cavities upon death. The thanatomicrobiome tries to investigate the total microbial communities including bacterial and fungi from all the body locations of decomposing corpses. These studies are important in providing evidence in medicolegal death investigations [39]. By doing this, the concept of human postmortem microbiome project (HPMP) has also been introduced which would create a consortium of research projects to identify and characterize the thanatomicrobiome and epinecrotic communities (e.g., epithelial tissues, body cavities, and the alimentary canal), relating to human decomposition with a potential of finding a state-of-the-art, more dependable, and molecular way of determining the time of death [40].

17.6 Potential to Be an Independent Field: Emerging Evidence

It has been shown that typical characteristics and pattern of the human microbiome might identify individuals and remain constant in the individual but differ from the others [7, 41, 42]. This means that individuals might be specifically and consistently identified using their microbiome. However microbiome-based identifiability is still a long way to go. Franzosa and co-workers have suggested a few means to achieve the target of identifiability using microbes. The identification of a “metagenomic code” that remains unique for an individual for a longer duration of time and stands true for a sizable population is the key [43]. Hence microbiome establishment, structure, personalization, and temporal stability are the standard terms and areas to work upon.

There are some untraveled avenues which start from microbial forensics. Emergence of antimicrobial resistance, prediction and prevention of future outbreaks, etc. are some of the fields wherein a surveillance system making use of the principles of microbial forensics can help. Antimicrobial resistance in microorganisms emerges naturally. However, antimicrobial exposure due to human practices in healthcare, agriculture, sanitation, industrial processes, travel, and other fields contributes significantly. Timely detection of pathogens harboring resistance can mitigate the onward transmission among individuals and among different geographical locations. Timely detection of pathogens like HIV, severe acute respiratory syndrome (SARS) virus, and pandemic influenza could have avoided the big health emergencies which we have witnessed in recent past. Similar is the case with the pandemic spread of SARS coronavirus in 2003 and H1N1 influenza in 2009 resulting in substantial economic loss. Microbial forensics can play a crucial role in such cases by checking the emergence in real time and suggesting measures to prevent transmission. However, a lot of investment is required to place such services at all vulnerable points or check points. Technological advances especially in the field of metagenomics have paved way for identification of potential human pathogens among other species and have attained good predictive power regarding transmissibility and virulence of the novel microbes.

17.7 Limitations

The science of microbial forensics is in its infancy and needs much more that what has already been done. A few things which needs to be taken care as preparation before we take this as a routine science are protocols and procedures for collecting specimens at the attack site, recognizing that an attack is occurring and diagnosing the disease, analysis of specimens in contained facilities, quality assurance and control. Although microbial forensics involve techniques or methodologies from basic laboratory sciences, the problems in question, processes engaged in and expected outcomes need more than that. There are efforts and continuous need for validating all the tools and techniques involved which should be acceptable to peers and stakeholder from scientific, legal, and policy making side. The optimization of methods to answer key questions pertaining to investigative and legal needs is a must to satisfy the criterion of acceptability. Meeting these challenges will allow the establishment of a complementary and reliable method to compensate for the lacuna of DNA fingerprinting and fingerprinting as discussed earlier.

Meeting the challenges needs the consorted efforts from global communities of workers from basic sciences, epidemiologists, forensic experts, medical experts, legal experts, and a big team of technology developers. The most reliable technique till date for microbial forensics is metagenomics—a culture-independent approach for identifying and enumerating microbes. Metagenomic have been an outstanding technique for sequencing the genomes of unculturable microbes, which represent the vast majority of microorganisms, particularly from environmental samples. However, technological advancement identifies the rare taxon is awaited.

As microbial metagenomics is undergoing a formative phase as a diagnostic technique, optimization of methods and their validation remain a challenge. Other aspects of this are the leadership role and generic availability of tools and techniques. Countries with major resources would be able to take lead in basic research, while the resource-limited setting may not be able to adapt microbial forensics owing to its monitory needs. Availability of equipment and techniques for rapid and precise molecular diagnosis is important for controlling and responding to the needs of microbial forensics. Till date, next-generation sequencing is the only technology that seems promising for microbial forensics. But the instrument and the reagents remain very costly as compared to biochemical tests and a few basic molecular assays being used in forensic laboratories. Moreover, expert workers and bioinformatics analysis of the huge data generated after massive parallel sequencing or next-generation sequencing require a devoted facility and expertise.

17.8 Conclusion

Microbial forensics may, in most of the cases, be associated with the detection of causal pathogen in cases of biological terrorism, but microbiome associated with individuals and objects used or touched by them at crime scenes may also be used as a tool in providing forensically relevant information. This science has got its diverse utility in the field of medicine, agriculture, trade especially in food articles, etc. as discussed in this chapter. The evidence and the literature available till date indicate a definitive linkage between an individual and microbial communities inhabiting that individual skin or other body parts. The science is progressing toward identifying these communities, next steps of which would be to see if these communities have something in common and that common character has something to do with the individual. Later work in this field may identify chemical entities and their microbial connections to land up to the final conclusions about signature communities of microbes and their forensic potential. However, we may have to continue our search to see those days soon.

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