

■ REVIEW ARTICLE

# Microbial Forensic: An Update on Advancement and its Applications

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## ABSTRACT

Microbial Forensics (MF) is an interdisciplinary branch utilized in various microbiological methods to study elaborately various pieces of evidence involved in criminal cases focusing on forensic attribution, bioterrorism, frauds, outbreaks, toxin or biological agent release. Microbial forensic investigations characterize nonbiological and biological (toxins, fungi, bacteria and viruses) evidence. Recently DNA sequencing and next-generation sequencing technology have given us the opportunity with advancement in genetics and molecular biology and have been applied in fields including disease diagnosis in forensics. Metagenomics is a culture-independent method to sequence microbial DNA which is collected from mixed community samples and environment. Several studies which shows applications for analysis of metagenomic sample for investigations in forensic science i.e., cause of death, detect time since death, biological fluid characterization, outbreak investigations, environmental samples and bio-surveillance. The major microbial diversity which is unaware of and so the present database does not accurately show the diversity which exists and thus various programs have started that sequence the reference genomes. Application of bioinformatics deals with the analysis of biological data and phylogenetic reconstruction. The analysis and interpretation of microbial forensic science data need to compare the data with a complete reference genetic database. Proper training and education in this field are essential for the scientists of the next generation to protect society from various causative harm which results from the act of biocrime and bioterrorism. Thus the development of various resources and infrastructure related to education must be aimed at practitioners of next-generation, policymakers, researchers and communities related to enforcement of the law.

**KEY MESSAGE:** This review provides insight into microbial forensic and investigations based on next-generation sequencing technology, metagenomics, and bioinformatics. With adequate training and education in this field, scientists can protect the society from various harms.

**KEYWORDS** | microbial forensic, microorganisms, forensic investigation, next-generation

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## INTRODUCTION

**R**ESearch in Microbial Forensic (MF) have made many changes concerning development, evolution and expansion, incorporation of latest technologies, various tools, and implementation of various capabilities of analytical nature encouraging the cumulative preparation and its response. Microbial forensics may be defined as the

scientific area which is given for explaining various shreds of evidence from the acts of bioterrorism, or the use of biological agents to kill people or make a person sick, or unknowingly microorganism release and toxin for purpose of imputation.<sup>1</sup> This field is employed in various microbiological methods to study elaborately various pieces of evidence

which are involved in various criminal cases particularly focusing on forensic attribution, ranging from frauds, bioterrorism, biocrime, bacterial outbreak spread from pathogens, or accidental release of agent of biological nature or/and toxin.<sup>2,3</sup>

Microbial forensic is involved in careful examination of suspected laboratory materials which are microbe-related found at the scene of crime suspected laboratory, for attribution to forensic science and plays a pivotal role for development and investigation leads. Attribution is termed as the sample characterization with highest specificity, which for microorganism is at the strain or species level, isolate level<sup>c</sup>. Usually pathogen outbreak observation and toxicology are not regarded as important elements of MF. As these topics are basic to MF, because pathogens emission can be purposefully or because of medical malpractice and by applying a reliable and careful protocol of surveillance for monitoring the pathogen which would give us invaluable information to differentiate from sudden and damaged spread of microorganisms (related with biocrime or bioterrorism).

In the past decades, the number of pandemic had been escalating.<sup>4</sup> During a bio terror attack, the biological agents are willfully released with the intention to kill a lot of people, and cause loss to economy, which is motivated by political, ideological, and religious beliefs.<sup>5</sup> The agent is often used as they are present in nature or engineered genetically and improved for mass destruction, results mortality and resistant to the presently available medicines or the vaccines. If we face the chances of attack by bioterrorist weapon it is important to identify the agent which is involved, to prevent panic among the population and also to contain the mortality and morbidity which is related to the spread of these agents.

#### **Epidemiological forensic investigations:**

Biothreats received renewed attention after the 2001 anthrax letter attack in the US. Not even a month had passed when 9/11 happened, which

was a bioterrorism act which was committed with the use of Postal Service of United States as a vehicle for dissemination to purposefully dissipate the spores of *Bacillus anthracis*.<sup>7,8</sup> The biological agents which can cause harm can be easily available, less sophisticated, easily distributed and relatively cheap in comparison to the various types of weapons to cause massive destruction of masses. The weapons of biological nature is a continuous warning to mankind for various biocrimes and bioterrorism cases.<sup>9</sup>

The center of investigations for microbial forensic to detect and characterize facilities related to both biological agents, along with the nonbiological evidence. Biological agents comprises of fungi, bacteria, toxins, protists and viruses. Evidences of not biological in nature, such as delivery devices, additives of growth media is useful forensics microbiology, to provide investigation leads which also helps to conclude the methods for manufacturing and also for dissemination of the non-biological evidences and the analysis which is an important part of microbial forensics.<sup>10</sup> Microorganism and its related toxin are preferable tools because are less costly compared to the cost to culture them, is easy to procure them if endemic or which occur natural way, only minute quantity of biological materials are able to cause death or spread infection. There are a number of species of microbe that might be helpful as useful threat from biological weapons such as (plant, animal and human pathogens) around 1400 microbes cause infection to mankind.<sup>11</sup> The microorganisms which are dangerous to national security and public health are listed in Center for Disease Control and Prevention (CDC).<sup>9</sup>

Toxins are produced by fungi, bacteria, eukaryotes plants, and *Botulinum toxin*, which are synthesized by *Clostridium botulinum*, which is a bacterium and a potent harmful agent which is dangerous and has lethal effects when consumed also in low doses.<sup>12,13</sup> There are several evidences of Phylogenetic studies which has been used in various law courts to give

relevant explanations regarding these crimes which involves infectious microorganisms. By constructing strain phylogenies and species and by utilizing the various information, such as disease transmission is concluded from an individual to another during infection and transmission of disease.<sup>13,14</sup> Outbreaks of disease is incident throughout the world and occur every year, and investigations in these outbreaks includes microbial forensics investigations and epidemiology. Public health and microbial forensics have similar interests regarding the genetic characterization and identification of various biological agents and how it can be dispersed in a given population.<sup>15</sup> A common path of microbial forensics and public health is to determine whether the transmission is accidental, intentional or natural. Epidemiology and forensics microbiology both are combined fields, which according to law focuses to attempt and individualize the toxin or agent and how it will be produced and dispersed by law enforcement concentrate and microbial forensic scientists.

#### **Detection and characterization with traditional methods and their drawbacks:**

The primary goal in microbial forensics is to compare the data received from various evidence samples to reference samples. The evidence can be from various samples which includes water, swabs, air filter, food, soil, water, samples related to clinical specimens (e.g. sputum, blood, tissue, stool, urine) in forensic microbiology. So, an analyst should have various methods for processing sample to meet the growing needs very large number of sample possible in the present scenario.<sup>9</sup>

Methods to detect traditional manner in the microbial forensics can vary from microscopy culture. The best way to detect pathogen is only by culture. Drawback of culturing is that it is not able to give proper resolution, beneath the level of species or genus, and as there may be lag time that is substantial, it is also not so effective particularly safety concern point of view of the of individuals. Around 99% of the microorganism are unable to be cultured

by present methods.<sup>16</sup> Biological evidences in addition to other threatening agents of genetic nature, like immune response of host, gives us a very valuable information related to investigation leads such as if a suspected perpetrator had taken antidotal substances or prophylactic antibiotics, decided by the manufacture, handling, possessing a agent of biothreat.<sup>17</sup> Traditional forensic evidence like human or animal DNA, fingerprints, hair and fibres can be analyzed. Immunoassays and culture are potent methods for sample screening, initial testing, nucleic acid typing is quiet resolving.<sup>9</sup>

#### **Emerging methods for detection and characterization:**

Traditional forensic evidence like human or animal DNA, fingerprints, hair and fibers can be analyzed. Immunoassays and culture are potent methods for sample screening, initial testing, typing of nucleic acid is quiet resolving.<sup>18</sup> MLVA [multi-locus variable number tandem repeat (VNTR) analysis] for the analysis of polymorphic regions.<sup>19,20</sup> The SNP marker detection approach is the use of microarrays, which consists of many large numbers of small oligonucleotide probe. Microarrays, which can be highly efficient characterization and screening tools, have been developed specifically for viral and bacterial identification.<sup>21,22</sup> The marker detection SNP approach which is microarrays use, which comprises of huge numbers of probe of oligonucleotide. Microarrays, which is very efficient characterization and screening tools, have been developed particularly for viral as well as bacterial identification.<sup>17</sup>

*Sequencing:* DNA sequencing technology has given us the opportunity to enormous advances in genetics and molecular biology. However in Sanger sequencing technology the disadvantage is its high cost, low throughput and difficulties in its operation.<sup>23</sup>

**Next-Generation Sequencing (NGS) technology:** The development of next-generation sequencing (NGS) technology, which has high-throughput capacity and low cost, has averted these problems to a

large extent, and the technologies which is applicable to many infields which includes disease diagnosis forensics, ancient DNA analysis and agrigenomics.<sup>24-26</sup> Non-Sanger-based NGS technology refers high-throughput DNA sequence technology. DNA molecules can be sequenced in millions or billions copies, thereby increase the amount of product to minimize and substantially the requirement of method of cloning a fragment which is used in sequencing by Sanger method. Based on loop array sequencing, includes second-generation sequencing, which analyses simultaneously a number of samples, which can study the composition of base of single DNA molecule in third-generation sequencing technology.<sup>27</sup>

(i) *Analysis of Short Tandem Repeats (STRs)*: Now a days, majority of forensic nucleic acid based tests employ capillary electrophoresis (CE)-based fragment analysis and PCR methods to identify changes in short tandem repeat (STR) markers. In forensic science nucleic acid applications based various technology used for investigative purpose in forensic science has provided DNA analysis as an essential techniques. DNA analysis in forensic science is confronted with highly degraded DNA sequence of contaminated samples less copy number, with high reproducibility and accuracy, with cost and time considerations.<sup>27</sup>

(ii) *Analysis of Mitochondrial Genome*: Recently forensic mitochondrial DNA (mtDNA) analyses mostly detects in hypervariable area the polymorphism. But in case of mtDNA which may be used as haplotype marker of genetic nature, addition to loci which is polymorphic in nature are necessary to elevate the power to discriminateto identify. So, NGS technology have the ability to help in the evaluation process and analyze theentire mitochondrial sequence.<sup>27</sup>

(iii) *Analysis of Y Chromosome*: In forensic molecular biology the markers of genetic nature which is assumed an important role and play pivotal role is located on Y chromosome. The male component of DNA mixtures need to be resolved unambiguously, commonly

Y-STRs which is used and present with high female background, or to create relationships of paternal side between different male individual. The two male individuals which is used in NGS technology had shared the identical ancestor around 13 generation before and because of this more than 10 million Y chromosome nucleotideswere comparatively studied.<sup>28</sup> Additional information present within the human genome may gives us an ideaof personal characteristics such asphysiological and physical characteristics, age and ethnicity.<sup>29,30</sup>

(iv) *Analysis of phenotypic and Ancestry inferences*: Previous studies suggested SNPs resembles hair and iris color with an accuracy of 90 per cent.<sup>31,32</sup> Evidences are there for which investigation on features of facewith the help of test onDNA and association were analyzed and legally approved with the results using face reconstruction.<sup>33</sup>

(v) *Forensic Microbiological Analysis*: forensic microbiology is current area originated by the Federal Bureau of Investigation (FBI) just after the attack by Anthrax held in USA on 18 September 2001. This area is decided on the accurate identification quick detection of microbes found in crime area of biological nature, which has the target to trace the microbe.<sup>34</sup> Terrorist attack by using microbiological forensic led to disastrous consequences therefore the microbiological forensic analysis had attracted a good attention.<sup>35</sup> With the help of sequencing by whole genome by the solid system, in which particular suspects by sequencing four strains each of *Yersinia pestis*<sup>36</sup> and *Bacillus anthracis*. To identify biological traces in a study of 454 sequencing systems using metagenomic analysis and deep sequencing which suggests that the technique is used to identify of forensic material of traces of traces of biological samples.<sup>37</sup> In another study they studied that the bacteria which is left byskin possess enough DNA information for forensic analysis<sup>38</sup> of human using NGS-based metagenomic method.

(vi) *Epigenetic analysis*: Various studies recently have suggeststhe epigenetic markers

can also have many uses in in microbiological forensic. Example, evidences suggests that markers of epigenetic may be helpful to differentiate monozygotic (MZ) twins, accurately determine the age and predict tissue type of a DNA donor.<sup>39,40</sup> Epigenetic approaches based on NGS technology include reduced representation bisulfite sequencing and methylated DNA immunoprecipitation sequencing, methylation beadchips and whole-genome bisulfite sequencing.<sup>41,42</sup>

(vii) Analysis of MicroRNA: Introduction of microRNAs (miRNAs) in forensic microbiology which are endogenous RNA molecules small in size and of<sup>18-24</sup> nucleotide lengthwise. Due to the small size, high tissue wide diverse expression and resistance to degradation they are useful for post-mortem interval (PMI) inference analysis<sup>43</sup> forensic body fluid identification, identification of species. In a study in 2009, miRNA profiling introduced to forensic science and found that 452 miRNAs were genotyped by quantitative PCR from forensic samples.<sup>44</sup> Another study shown that the expression levels of 718 miRNAs in semen, venous blood, saliva, vaginal secretions and menstrual blood were analyzed on a microarray. Out of these 14 expressed miRNAs was recognized, which might be act as candidates potentially identified for examination and to identify of body fluid. By the use of the technique NGS, sequences of millions of miRNA is analysed rapidly thus provide a potential tool in analysis in forensic science.<sup>36</sup>

**Metagenomics:** In metagenomics application to sequence the DNA obtained from complex and environment and mixed community samples. It's a culture-independent method for microorganisms study and analysis collected from environments such as soil, water and human-associated samples.<sup>45-47</sup> Species are not able to be cultured for identification<sup>48</sup> and around 1,030 bacteria exists on earth. Many studies have shown the applicability of metagenomic sample analyses for forensic investigations like for human identification,

reason of death, to know the time of death, for biological fluid characterization and identification, for disease outbreak investigations, for environmental samples, and for public bio-surveillance.<sup>49-54</sup>

Additionally to detect target pathogen from the desired samples for metagenomic analyses, for biodefense purposes and epidemiological study in microbial community profiling in forensic microbiology utility. The types and conditions of sample is encountered in a microbial forensic investigation are fluctuating and may be added with different nucleic acid which makes the methods of detection quite challenging. Microbial forensics focuses comparative analyses and to detect and compare and analysis of important pathogen which detect agent very rapidly from pure or homogeneous samples. There are two primary ways for metagenomic sequencing, Whole-genome shotgun sequencing (WGSS) or targeting the 16S rRNA gene. Among these two methods, WGSS is more desirable for microbial forensic metagenomics analysis as species level resolution.<sup>9</sup>

**Data analysis and interpretation:** Bioinformatics deals with the methods to apply various computation method which analyses data biological in nature, such as massively parallel sequencing (MPS) data which is required in interpretation of data, statistical analyses, reconstruction phylogenetically and representation of data visually. With the evolution of MPS and the onslaught of data sequence many data management systems and bioinformatics software tools had been developing for metagenomic assembly by utilizing software tools for, taxonomic classification, phylogenetic analysis, entire metagenomic analysis pipeline and database analysis and management systems.<sup>55-59</sup>

**Challenges in result interpretation and validation of Microbial Forensics:**

Microbial analyses which is of ancient origin is one more branch of forensic related targets in expanding the limits to analyze samples challenging in nature. The past history

suggests that spread of disease has led to the deaths of many people across the globe. In few of epidemics the actual cause or the agents causing is known, however for some past epidemics the causative agent was controversial or stayed a mystery.<sup>60</sup> In recent had shown to improve extraction technique with highly sensitive detection assays, and with various sequencing technologies have been developed enhanced the ability to characterize genetically these ancient pathogens from skeletal remains and other sample types. Because of damaged properties and fragmentation of ancient DNA, novel library strategies and preparation methods and bait using comprises of nucleic acid sequences which was used.<sup>61,62</sup>

Proper interpretation and evaluation of forensic microbiology evidence is very important and cannot be ignored to establish confidence, in scrutinizing the legal system to making critical decisions and policy. Though, consequences were devastating which occur we can prevent it which depend on results of microbial forensic. The interpretation of standard guideline in the field of microbial forensics is lacking, thus the analysis of phylogenetic trait has association which support them and has admitted as evidence in proceedings of legal and criminal cases successfully in United States also abroad.<sup>12</sup> Reconstructing phylogenies has been used as a Microbial forensic tool is used to reconstruct phylogenies convicted individuals in cases of intentional infection with RNA viruses.<sup>13,14</sup> The girlfriend of Dr. Schmidt was injected with with a mixture of hepatitis C virus (HCV) and HIV infected blood his two patients.<sup>63</sup> Data study of a particular region can plays an important role in investigation of forensic microbiology investigation to check the presence of a microbes probability being due to a intentional outbreak or natural means. In 2009 injectional anthrax of many cases was diagnosed for population taking heroine in Scotland. In Scotland B. anthracis was not endemic. WGSS and SNP genotyping was used to determine origin and strain of the B. anthracis spore, that was introduced in Turkey

or its surrounding area.<sup>64</sup>

Databases are an very important tool in forensic microbiology. Microbial forensic interpretations with analysis require the comparison of genetic data to completely characterized references available in databases. Databases must includes as possible as many strains of species, in addition to near-neighbors and other microorganisms representative of a range of phylogenic organisms. Metadata are the information associated with a given sample like date of collection, collection site location, tissue source, extraction and sequencing methods, virulence, assembly and annotation methods and can be used to determine endemicity and other information. Metadata are important to epidemiological investigation which provides information to aid in therapeutics, source tracing, and essential to microbial forensic investigations provide a valuable information for investigative purposes.<sup>17</sup>

The microbial diversity remains in majority and still unknown so the present database does not reveal the range of actual diversity which occur and programs has started to know the reference genome.<sup>65</sup> In the area of microbial forensics the main target is on microbes which are pathogenic and infectious many of which are sequenced along with continuous improvement in MPS, so the total number of microbial genome which are available increases everyday. The Human Microbiome Project (HMP) consortium started the work of sequencing new reference genomes to increase and interpret disease state and health conditions of microbiome. 100K Foodborne Pathogen Genome Project and Human Microbiome Jumpstart Reference Strains Consortium, 2010 targets to sequence 100,000 different foodborne pathogen which is helpful in various epidemiological related investigations.<sup>66</sup>

Role of education and training for awareness and implementation: To understand the forensic microbial field is required to determine which evidence is collected, what are the safe and proper methods related to collect and preserve, techniques to analyze the evidences,

result significance, various supports to identify a prosecution perpetrator. Education along with training given in the field is necessary for preparing next generation scientists.<sup>67</sup> The applications, scientific bases, advance interpretation and lesson which is acquired by those who had actively involved in gaining knowledge on microbial forensic required to document and transfer the next generation scientist and decision maker to impart the society with better protection from potential harm resulted from act of biocrime and bioterrorism. Thus development of infrastructure of education and various resources should target to successive generation of practitioner, various research, diverse elements for the policy, and law enforcing communities.<sup>67,68</sup>

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#### CONCLUSION

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Forensic microbiology which is interdisciplinary branch which includes various law enforcement, scientists, public health, intelligence community, decision and policy makers. All of them help to give us a system which helps us to and give us protection from various disease outbreaks that occur naturally or epidemics and the act of intensely occurring biocrime and biological terrorism. Recent development in various methods at the molecular level, particularly next generation sequencing (NGS) technology and methods of whole genome sequencing, gives tools to forensic microbial scientists to find out crucial and important information with reduce them the cost than earlier. For the researchers it is imperative in the forensic

microbiology to continuously carry on with their novel research in areas such as method and comparative genomic and bioinformatics software development, and to increase the databases. Continuous updates for evaluation and to quality control and assurance in forensics microbiology must be maintained and practiced up to their standard. Evaluation and result interpretation in forensic microbiology investigations should follow the right criteria with appropriate validation. Recent genome-based data and technology, databases with expanded reference genomes, validated method and endemic data together contributes to the proper interpretation of result in a forensic microbiology investigations. Confidence of results is based on high quality are important since forensic microbiology interpretation have huge impacts on our society, political policy, regarding economy and safety. Many challenges evolved and continuously exists with time in forensics microbiology but introduction and updated technology implementation and regular communication across the scientific, intelligence, law enforcement, policy makers and public heal this expected to contribute towards the advancement of the forensic microbiology. **IJFMP**

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*The authors declare that there is no commercial or financial links that could be construed as conflict of interests.*

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