

■ REVIEW ARTICLE

Microbiology as Forensic Tool in Investigation of Bioterrorism

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ABSTRACT

Bio weapons were always a known part of wars and very much acknowledged form of terrorism even from Ancient times. Microbes can be used as good arms as their mass production does not requires much skills, quantity and time, along with that they can grow well in numbers from a single cell. Highly pathogenic micro-organisms and their toxins are always in concern as they were used as Microbial-terrorism, one of the much known examples of bioterror is spores of Anthrax attacks at United State in 2001. The word 'microbial-terrorism', itself Indicates, the use of pathogenic microbes, to be dispersed among the group of people/ in certain areas/ or certain countries as a fatal disease, which act as epidemic or some time pandemic. In view of these Forensics investigation, microbiology plays an important role such as sample handling, configuring, tackling, investigation, and validating of bio crimes. The main purposes of this review on Microbiology as forensics tool, is to explain how microbiology helps in investigation of bio-terrorism, and also to suggest the systematic scientific diagnoses of bioweapon. With this knowledge, people and nations can be secured from emergent bio-threat of future.

KEY MESSAGES: Microbial forensics is one of the most essential and significant technologies for detecting bioterrorism and bioweapons. As microbes are associated with decomposition that can impact the investigation results of autopsy, toxicological studies, and histological views. As a result, microbial-forensics can always be of tremendous assistance in criminal investigations.

KEYWORDS | Microbiology, bio-weapon, bio-terrorism, microbial- forensics

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INTRODUCTION

MICROBIAL FORENSIC SCIENCE GAINED LIMELIGHT after the attack of Anthrax spores via postal service in 2001 at USA.¹ Microbial forensics, is combination of two field which deals with cause of crime but with use of microbiological tools. This field mainly comprises of all the techniques used in microbiology, such as biochemical reactions, normal flora identification, culturing, molecular assays, genes, phylogenetics etc. in addition to that, basic principles of forensics, such as collection of evidence, chain of custody presentation in court etc., are also a part of

that.² Few of the similar methods used in DNA studies of humans are also been explored to microbial analysis such as single nucleotide polymorphism (SNP) analyses, microsatellite and minisatellite loci typing, RT-PCR etc. Bacterial DNA fingerprinting is much more difficult than the humans DNA fingerprinting, because bacteria are haploid, and can reproduce much more rapidly and often undergo recombination gene transfer.² The diversity of bacteria can be studied by use of phylogenetic tree, which give knowledge for relationship of different genus and their spp.² In other words,

we can say Microbial forensics is the branch of science using scientific methods for evaluation of evidence from hoax, bioterrorism outbreak, bio-crime such as assertion of a bio-products or toxins, etc. Provenance of microbial evidence comprises exploring an allied source or culprit or set of entities to the highest degree of possibility. The arena of microbial forensics is based on a various fortes of biological sciences (e.g., forensic sciences, microbiology, biotechnology, genetics, biochemistry, immunology, bioinformatics, molecular biology, population genetics, epidemiology, etc.) and along with that law, public health, the law enforcement, policy and intelligence communities are also part of that.³

After the outbreak of Anthrax bacillus spores in the US, Microbial Forensics and Epidemiology are collectively working for determination of any type of outbreaks (some are summarized in Table 1), whether it is accidental, natural, or intentional. In addition to that, predefined forensic approaches such as analysis of DNA, fingerprinting, trace elements or materials, and handwriting examination, are also used for the investigation of crime.³

Microbial forensics is a noble field of research, and still in initial stages of expansion; because of this it is still going through lots of ethical and scientific trials to find a strong base for its existence. This fact is well known that the Illicit

use of microbial or biological products, having potential hazard to environment, humans, economy, global peace and world-harmony.⁴ As we know, the “microbial Forensics” principally deals with crime study and legal proceedings, maybe It may not crack the entire case, but definitely may lead the investigator in a correct direction.⁴

Bioterrorism is a word used for microbial agents and their toxic products which utilized for causing harm to the humans or other living bodies. The use of microbial agents in wars is being rehearsed from prehistoric eras. For example, usage of toxin coted arrow, putting poison in water and food supplies and knowingly dispersing fatal microbes (*Clostridium tetani*) to injured soldiers in clothings such as blankets. These infectious agents are proficient to lead or cause pandemics, epidemics or large outbreaks in a short span of time and the best example we have seen is outbreak of Corona virus.⁵ The bio-weapons are not only a threat to humans, but also to plants, animals, economy, agriculture, country, and to the whole ecosystem.⁵ Bio-attacks can be classified as either obvious or secret and the main difference among the two is obvious attacks are normally identified immediately, whereas covert attacks take a long time. On the other hand, covert biological attacks are much difficult to determine than the obvious attacks.⁶

S.NO	CASE STUDY	YEAR	REFERENCES
1.	Specially characterized strain of Staphylococcus aureus from paraphernalia to track drug networks	2000-01	25
2.	Reports, origin of Bacillus anthracis from heroin users in Scotland	2009	12
3.	The Origin of the Haitian Cholera Outbreak Strain	2010	26
4.	Phylogenetic analysis to provide evidence against Richard Schmidt that he intentionally injected HIV/HCV-contaminated blood to his girlfriend.	1994	18
5.	Investigation compared different Ames strain morphotypes to determine the origin of the source material released in the 2001 anthrax attacks	2001-02	1,13
6.	Escherichia coli O104:H4 outbreak in Germany in 2011	2011	27
7.	Initial report of molecular tracking of HIV infection from dentist to patients after invasive health care procedure		
8.	Phylogenetic and molecular clock analysis to provide evidence that a Spanish anesthetist infected 275 patients with hepatitis C virus	1998	29
9.	Ebola outbreak	2014	11
10.	Parvovirus B19 characterization from skeletal remains revealed the likely origin of World War II casualties	2015	30

Table 1: Cases of microbial forensic along with epidemiological (in interest to forensics) investigations.

Standard Protocol of Investigation

It is very much required to identify natural outbreaks and intentional attacks. And for these identifications, microbial forensics comes into the picture. Separating intentional from non-intention biological outbreaks needs an in-depth understanding of normal pathogens and their epidemiology. Therefore, a substantial method for detection, awareness, and surveillance measures are required. The microbial-forensics collects the evidences and provides the information about such cases (intentional attack).⁷ Once the biological samples of evidences are collected and preserved, then it is sent to a specific laboratory (depends on the type of biological samples) for further analysis. In the lab, a set of analytical apparatuses are present to provide required assistance in characterization and classification of the evidentiary material. As microbial forensics mainly focuses on chasing microbiomes with location and individuals, different approaches may be applied, and it is totally liable on the type of specimen and the attack. In an obvious attack, for example, weapon, package, and allied materials, along with forensic evidence such as nail, hairs, fingerprints, fibers, etc.) may be evaluated. On the other hand, in a secret attack, the evidences are restricted to medical antiquities, isolates from victims and diagnoses. In 2001 anthrax attacks, a blend of both methods was applied in the investigation and these shows that an investigative analytical plan for biological attacks may requires several and diverse approaches or strategies. After identifying the cause of bio-crime, microbial-forensic expert proceeds for a more investigation for example, detail study of bioweapon (pathogen) by custody of evidence. The protocol for investigation or evidence collection comprises, collection and preservation of samples, and then investigation with respective techniques at National forensic laboratories.⁸

Tools of Microbiology

Numerous of methodologies already exist, but molecular biological tools are conspicuously

absent in the selection criteria of the microbial forensic scientist. Relative genetic categorization of pathogens (bioweapon) is a key segment of their provenance process. There are a number of genetic markers and approaches that permit accurate and high potencies of specificity in classification of microbial diversity. For forensic determination, rapid assaying markers of high quality are in use for providing better lineage-based evolutionary analysis, defining strain and species of microbes. As viruses, bacteria, and some fungi reproduce by asexual means, their genomes are much constant and can be clonal too. Therefore, it may be uninformative for differentiating samples by genetic analysis as it is proficient in human DNA identification testing. Uncertainty in microbial genome is more than the humans due to limited database, unknown diversity, huge manipulations and limited genetic analysis.^{9,10,11}

Genetic analysis methods used by epidemiologists to track infectious disease outbreaks employ interpretation guidelines to evaluate the results and to determine case relationships in epidemics. Forensic analyses can often use the same or similar methods but may require additional criteria, such as identification of individualizing characteristics for higher resolution source attribution.^{12,13}

Hence, genotype variation analysis, for example, polymorphism and DNA fingerprints need to be stressed. By these techniques, genomes from specimen (evidentiary sample) need to be compared to a standard reference specimen for analyzing if they both are belonging from the same source and also shares the recent lineage. Few techniques which are used for human DNA studies have been also explored for studying microbial investigation, such examples are single nucleotide, minisatellite and microsatellite loci typing, polymorphism analyses, and RT-polymerase chain reaction (PCR). For imputation of bioweapon (causative agent), DNA typing method is to be considered as enormously significant technique and the first step in the analysis is the extraction of DNA, for that various biological tools and biomarkers are

present to assist the analysis, some examples are, sequencing, microarray analysis, pathogenicity array analysis, single nucleotide polymorphisms (SNP) characterization, 16S rRNA sequencing, variable number of tandem repeats, antibiotic resistance gene characterization etc.¹⁴

For the evaluation of the microbial pathogenicity and for improved determination of microbial strain or sub-strain various methods are used, such as immunoassays, bio-functional assay, peptide or protein-based assay, mass-spectrometry.¹⁵

The traditional physical evidence that described microbial forensic specimens are: physical morphological characteristic of microbes which were used as bio-weapon; isotope analysis mainly used for determining origin and age of microbe; further microbial Identification at species and subspecies level can be done by usual physiologic methods such as serotyping, fatty acid composition and phage typing; the sign to origin to the weapon or technique used to prepare material from the residue of growth media attach to the microorganisms; addition of stabilizer to the samples for preparation: time, year and location of the sample need to be recorded and labeled; along with all geological normal flora need to be studied and IgG and IgM antibody reactions need to be analyze for determination of recent exposers to microbes.¹⁵

DNA fingerprinting performed for bacterial genome is much more tough than the humans one, because bacterial species numerous in number and all need to be considered. In addition, bacteria they reproduce very fast and are haploid too. Most of them reproduce asexually, and endure recombination and horizontal gene transfer.¹⁶

DNA microarrays, also called biochips and are among the popular selections because it can precisely detect quantity of causative agents in a short span of time. Microarrays comprehends numerous of DNA sequences which may help in forming complementary sequences with suspected bioterror samples and evaluate it for its specificity.¹⁷

Matrix aided laser desorption ionization assay has been effectively explored for RNA, DNA characterization and protein studies at gene level. These approaches help in analyzing of subtyping of microbes.¹⁷

Comparative genomic sequencing: this is another valued means for identification of microbes. Though, it's not that fast as other methods but at the same time has the potential to deliver more specific and detailed information about the microbial strain.¹⁷

2D Spectroscopy: this is mainly working on the principle of optical and infrared spectral regions emphasizes on the technically scientific challenges faced by microbial forensic investigations. This technique helps in the visualization of time dependent morphological variations occurs in biological specimens, which may range from liquid to solid changes, new findings, and vitalphysio-chemical changes etc.¹⁸

Determination of ratio of stable isotope: this technique mainly helps in identification of molecular markers carried out by bacterial species. Stable isotopes of nitrogen, carbon, oxygen and hydrogen, can possibly provide the geo-location or medium in which bacterial species were grown in.¹⁹

The culture media provided for growing the bacteria has unique association with these stable isotopes atoms as, these isotopes are part of their molecular synthesis. The variation of Isotopes in the bacterial cell can be determined by the constituents, used in culture media used for their growth.^{20,21}

Applications of Microbial Analysis in Forensic

- Help in distinguishing natural, deliberate and accidental outbreaks with high level efficiency and short span of time.
- Aids in identification and characterization of microbial strains at subspecies level along with their normal habitat and pathogenicity.
- Help in rapid evaluation and development of novel microbial based forensic diagnostic approaches with capability of detecting numerous of parameters associated with

- crime.
- Sampling of bio-weapon based on microbial stains.
 - Forensic aspects of microbial crime need to be characterized by microbial based basic procedures.
 - Help in understanding microbial diversity and flora endemism for assessing the information about the purpose of an attack along with effort may have been developed or perpetrated, or how perpetrators may have exploited the microbial background.
 - Help in determination of causative agent and tries to give the answers of questions such as how the microbe and crime was associated; how should scientific and legal significance be determined and supported when the causative agent is a minority constituent in a “probative sample; how much of the threat agent of interest must be contained in a sample to be considered significant.
 - Help in determination and characterization of causative agents other than genomic methods such as omics (e.g. proteomics) and approaches such as multitarget examination of culture media.
 - Help in forensic based characterization and evaluation of biological toxins. In addition to those credentials, it can also evaluate the development of aid rule-in/rule-out determinations.
 - Outstandingly dropping the “discovery-to-decision” timeframe, across all bio-threat agents, with highest probative value and assurance. All the answers in forensics are integrated with the decision process.
 - It helps in validating even very low-level analytics (very small quantities of a target analyte) in an operative scenery. Capable of evaluating precise level analyte (e.g., DNA) might be made up of canonical single-nucleotide polymorphism (canSNP) or other isotope signature or at the few- or even single-molecule(s) level.
 - It provides international data-sharing forums along with quality and nomenclature standards; these are vital, and help as reference for microbial-forensic analysis.
 - Help in determining, how to evaluate and quantify with certainty and report whole-genome–sequencing contrasts achieved throughout the forensic analysis for example, associating an evidence “profile” with a reference specimen that may have a direct link or common ancestor. Sequencing errors along with other factors will probably inflate variation between specimens, which may create a degree of uncertainty.
 - Ensures the eminence of sequence data and the outcomes of bioinformatics analyses as high as possible. Factors that affect data genomic data representation is sequencing errors; uncertainty about databases (e.g., interpretations based on available data, including metadata), criteria for comparisons (similarities, different, inconclusive etc.), and the consistency of proficient perceptive (which comprises formulation of well-defined hypotheses, and evaluating techniques), for calculating the mass of microbial forensics evidences.
 - Help in standardization of bioinformatics software, which help by ensuring the proper analysis, and all based on previously assessments and comparisons of technologies can be made effectively?
 - Avoiding the filtering of data on the basis of individual preferences and bias.
 - Instituting processes to inform decision makers in a way that ensures that the science is properly understood. Many nonscientists who make decisions based on forensic science
 - Microbial forensics results that are very informative, have high confidence, and are rapidly obtained—and perhaps better leveraged with other capabilities—could enable investigators to manage risks so that energy is dedicated to anticipating and preparing for an event rather than reacting to a surprise, scrambling to mitigate consequences, and seeking attribution.
 - It also helps in studying medical and public health issues.^{17,22,23}

Legal Steps In Microbial Forensics Investigations

Microbial forensics are well known field for tackle the bio-crimes. For better understanding, example (hypothetical case), one microbial agent sustained or detected from the crime scene, is isolated and characterized and have some reference to be associated with the crime. Via a well-established scientific assay, the origin of the agent can be traced; either it is belonging from any laboratory or a person. When the lab or person's profile is matched, they will be summoned by investigatory bodies. Then judicial system has to prove their involvement in bio-crime act, and their motive of crime too. Then it is also required to provide the details about the origin of microbial agent. Some time, it might be impossible to verify the fact that same genotype of microbes exists in nature. In this hypothetical case, all efforts to prove the criminal activity may be unproductive unless strong statistical evidence is are gathered with unique signatures from the microbial agent and then only the entire purpose of doing it may be jeopardized.

In comparison, there are lots of similarities between human forensic and microbial forensic DNA analysis, such as use of qualitative conclusions of test results, population databases, and application of quality assurance/quality control practices, but differences do exist, for example, database size, protocols, contents and the analysis techniques. Contrasting an epidemiological survey, in microbial crime the evidence and samples should be preserved till the approval of the reports, that need to clearly show, whether the sample belongs to natural outbreak or holds as an evidence for judiciary purpose.^{5,6}

Scope of Microbial Forensics in India

As the United States is well known for technically advanced technologies for investigation of bio-weapons by using microbial forensics, many other countries are also investing and pursuing R&D for the development on microbial forensics. In the same context, India is also exploring their potential in microbial forensics

and it is incredibly vast. In India, government bodies such as National Microbial Forensic Laboratory act as a knowledge center composed of genomic databases of microbial flora, methodology based on microbiology, advances in forensics methods, SOPs, evidence assays such as bioinformatics, fingerprinting, and genomic standardized tools. These bodies also maintain the strong partnerships between the existing government, the laboratory scientists and investigating officials. Along with that they also monitor the quality control and validation of novel techniques and assays.²⁴

CONCLUSION

For the prediction of bioterrorism, microbial forensics has great value because it uses numerous of biological techniques for identification of natural and manmade bioterrorists by means of both molecular genetics and non-genetic technologies. RT-PCR, whole genome sequencing and sequencing of 16S ribosomal RNA, are molecular genetics based technologies used for causative microbial agent's characterizations and on other hand, non-genetic techniques are from the physical sciences, such as electron beam-based methods, mass spectrometry and, etc. can be used for evaluation of physical properties of causative microbial forensic evidence, for example, the existence of additives for enhancing the dispersibility of agent, or physical signatures from the location where the agent is originated from. By using these technologies, it will be easy for the investigator to analyze the threats more easily, and in short span of time. As Microbial world have good potential to be applied in forensic investigation, because they are ubiquitous. But at the same time is very important to develop consistent and reliable methods for evaluating microbes and their ecological habitat, because they can provide both temporal and spatial evidences applicable to investigation. Recent advances in microbiology have substantial inferences for illicit investigation and medico-legal death studies. For the microbial based evidences

studies, a development of reliable protocols is must and should also have properties like high quality, reproducibility etc. The advances in microbiology, directly or indirectly helps in forensic studies. As micro-organisms are involved in decomposition, they can always influence the investigation results of autopsy, toxicological studies, and histological views. Similarly, the investigation of postmortem bacterial translocation using biological-sciences based modern techniques is of countless importance. This is always recommended that forensic-microbiologists need to work in collaboration with forensic entomologists and anthropologists. **IJFMP**

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Conflict of Interest:

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