Environmental Microbial Forensics: How Hidden is the Truth?

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Abstract

The human society has witnessed bioterrorism and bio crime since biblical times. In the year 1155 A.D., Emperor Barbarossa poisoned water-wells with human bodies in Tortona, Italy. Since then, several instances of the use of pathogenic microbes to spread disease, have been witnessed to conquer nations or to practice terrorism. Easy availability of pathogens and involvement of low-cost methods have increased such instances. Managing risks and threats of bioterrorism and bio crime and strengthening biosecurity have become challenging tasks for governments and law-enforcement agencies. Microbial forensics is an arena of forensic science which applies the science of microbiology to forensic investigations. This field focuses on the characterization of evidence recovered from bioterrorism acts, bio crime, hoax or any inadvertent release of pathogens and biochemicals into the civilization. The forensic microbial investigation is like a routine forensic investigation as it also includes crime scene investigation, chain-of-custody, handling and bagging of evidence, analysis of evidence, interpretation of result and presentation in court. Different molecular biology-based assays such as nuclear acid amplification techniques, MLST (Multi Locus Sequence Typing), VNTR (Variable Number of Tandem Repeats) can be used to identify the microbial strains in the investigation process. This chapter focuses on the role of microbial forensics in enabling the law-enforcement bodies to deal with the menace of bioterrorism and bio crime systematically.

Keywords

Bio crime · Bioterrorism · Microbial forensic · MLST · Pathogen

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4.1 Introduction

Investigating officers, victims and people who are known to the victims usually want to identify the person who committed the crime. Physical evidence recovered from the crime scene plays a vital role in the investigation. Handwriting identification, DNA analysis, fingerprint comparison, body-fluid identification, analysis of drugs and poisons have been well established in the field of forensic science (Budowle et al. 2005a). In the twenty-first century, the ability to manipulate and disperse pathogens increased which led to a profound concern regarding the potential use of microbial pathogens and their toxins for causing significant harm to the plants, humans and animals. Such types of threats can be identified and solved using forensic science techniques. One of the significant threats that can affect humankind is an outbreak of a severe disease either naturally or intentionally. Such type of event can create havoc in the society resulting in death or harm and also disruption to the economy. There are numerous bacteria, viruses and fungi that can cause serious health issues to humans, plants and animals. Using them as a potential source of bioweapon can disrupt the economic stability and political activities of a state or country. Easier access to technology has increased the likelihood of using bioweapons for creating mass destruction. In some journals, it has been cited that the use of bioweapon is more economical as compared to the traditional weapon (Murch 2003). Thus, bioterrorism poses a significant challenge in society. There are numerous examples of bioterrorism incidents in history using a pathogen to create disruption. Anthrax letter incident in 2001 established the need to strengthen homeland security and introduce forensic science approach for externalization and dissuasion (Budowle et al. 2005b).

Apart from bioterrorism, pathogens and toxins can be used to commit bio crime. Bio crimes are similar to traditional crimes in many ways, as the main focus is to harm an individual. In traditional crimes usually, gun, stone or knife is used while in bio crimes, a biological weapon is used to harm an individual or inflict an injury. Most of the pathogens that were used in the past to commit bio crimes were less lethal, for example, *Bacillus anthracis,* smallpox and Ebola, but they were easily accessible. During the fourteenth century, the European plague epidemic started as Tartan soldiers used dead bodies of their fellow soldiers suffering from plague to create havoc and invade in the walled city of Kaffa (Derbes 1966). It is impossible to anticipate the next biological agent that can be used to create damage or for illicit purpose.

Some harmful microbes are grown by providing the required environmental conditions while some are grown in the laboratory conditions. A virus can quickly spread from one person to another in a short period and cause infection to millions of people so that there is an economic loss to that country. Intentional use of microbes for killing people has been reported in many countries.

In microbial forensics, the epidemiological issues are to be identified and characterized for a specific disease caused by a pathogen or their toxins. Also, the forensic scientists analyze the mode of transmission of the pathogens and any changes that have been made intentionally to change their effect against the humans,

animals and plant materials (Budowle et al. 2005b). The development of microbial forensics has been slower, relative to other disciplines of Forensic Sciences. Thus, it is considered as a minor program majorly in a variety of government agencies. Over the last few years, an acute awareness has developed regarding the threats of pathogen and toxin weapons which indicate microbial forensics has become necessary.

4.2 History of Pathogens

Biological agents have been used since the past centuries for spreading infection or for attacking the population of one's country. Anthrax caused by the bacterium *Bacillus anthracis* is used for biological crime due to its physical properties and virulence factors. Several countries have tried to use anthrax as a biological weapon in the military due to its potential (Pohanka and Kuča 2010). Anthrax is a rare bacterial infection caused by inhalation, ingestion, or if one comes in contact with the endospore of *Bacillus anthracis*. This bacterial species usually cause disease in herbivorous mammals and human beings who live near animals. Inhalational anthrax is a rare case, but a few cases related to inhalation anthrax were reported in the United States of America in 1978 (Aggarwal et al. 2011). In Russia, a military biological weapon facility in Sverdlovsk accidentally released the endospores of anthrax, and the case of anthrax appeared in humans that were even 4 km far away from the site. At the same time, some animals were also infected that were staying 50 km away from the site. By this way, we can say that pathogens can travel over long geographical distances. Globally there were two thousand cases due to anthrax, but only two cases were reported from the United States (Franz et al. 1997; Pohanka and Kuča 2010).

Plague is a contagious bacterial disease caused by *Yersinia pestis*. It is a zoonotic bacterium and usually found in small mammals and their fleas. There are three forms of plague infection in humans (Stenseth et al. 2008).

- If buboes are formed, then it is known as Bubonic plague,
- If the infection spreads to the lungs, then it is known as Pneumonic plague,
- If the infection spreads to bloodstream leading to systemic infection, then it is known as Septicemic form.

A common form of the plague is the pneumonic plague that killed over a million people across the globe. Many American army sailors were killed during the world war as they were moved to Boston from Philadelphia (Christopher et al. 1997). After 4 days, many people got infected and were hospitalized. More than one-quarter of Europe was infected. Usually, influenza kills the infants and elderly, but people who died due to this disease were mostly young men and women in their twenties and thirties. In other cases, the carcasses of animals and humans were used to contaminate the water supply of enemies. During the final months of the Second World War, Japan had planned to use plague as a biological weapon against United States

citizens during Operation Cherry Blossom. Japan had planned to execute this plan on September 22, 1945, but it could not be executed due to the surrender of Japan on August 15, 1945 (Baumslag 2005). In 1972, Biological and Toxin Weapons Convention was signed by US, USSR, UK and several other nations to ban the development, production and stocking of microbes and their poisonous products except for amounts needed for peaceful research (Ligon 2006). This convention came into force on March 26, 1975 and is a multilateral treaty of infinite duration. One hundred sixty-five countries signed it. The significant terms of the treaty were to ban the stockpiling, acquisition, development and retention of toxins and biological agents of "types and quantities" that have no justification for protective and peaceful use. If any state after entering into the convention possesses any biological agent or toxin, then they have nine months to destroy it or divert it for peaceful use (Working 2001).

4.3 Pathogens in Microbial Forensics

4.3.1 Categorization of Pathogens in Microbial Forensics

Certain properties make microorganisms useful as a biological weapon. Some of these properties are as follows:

- easy accessibility
- culturability
- large-scale production capability
- stability during the production process
- toxicity
- virulence
- ability to retain potency after the production process (Greenwood 1997).

Based on the etiology of the disease and mortality, pathogens have been classified into three categories:

Category A: Pathogens that have been placed under this category show a high mortality rate and cause significant impacts on public health. In most of the cases, initially, a wild animal is infected and then the pathogen transfers to the human population. For example, *Clostridium botulinum* found in canned foods, *Yersinia pestis* found in rats are category A pathogens.

Category B: Pathogens that are placed in this group have less mortality rate than the category A pathogens. Examples: *Rickettsia prowazekii*, *Coxiella burnetii.*

Category C: Pathogens placed in this category have the potential for high mortality and morbidity rates and can cause significant health impacts. Example: Nipah virus, Hendra virus.

Some of the common viruses that have caused havoc in the health care sector are:

4.3.2 Nipah Virus

It spreads through bats and pigs. The people who are living near to the pigs are infected with the disease. It was first observed in Malaysia and Singapore (Paton et al. 1999). It can spread from one person to another and from animal to humans. It is a type of RNA virus from the genus Henipavirus. It is a newly emerged virus, so there is no treatment for this virus. It can be prevented by avoiding exposure to bats, infected pigs and by avoiding drinking raw date palm sap. Around 50% to 70% of the infected people died due to this virus since there was no cure. In India, it first spread in the state of Kerala. This virus has been named after a village in Malaysia, i.e. Sungai Nipah, where pigs were infected and later on many people got infected with this virus in the year 1999, to stop the spread of this virus, a massive number of pigs were euthanized (Syed 2018). In India and Bangladesh, the disease spread from one infected person to another. Symptoms usually appear after 5–14 days which can be fever, headache, drowsiness followed by mental illness.

4.3.3 Hanta Virus

ORTHOHANTAVIRUS or Hantavirus is a negative-sense RNA virus. It mostly infects rodents. When humans come in contact with infected rodent urine, saliva or faeces, they get infected. Hantavirus is named after the river Hantan that is present in South Korea.

4.3.4 Brucella

Brucella is the causative organism of brucellosis. It is a highly contagious zoonosis and is caused through unpasteurized milk or uncooked meat from infected animals. It is also known as Undulant fever, Malta fever and Mediterranean fever. It is a small, gram-negative, non-motile, non-spore forming rod-shaped bacteria. Since the twentieth-century Brucellosis has been recognized in humans and animals. Brucella species was used as a weapon in certain countries in the mid-twentieth century (Leitenberg 2001).

4.4 Role of Microorganisms in Forensic Investigations

Microbial forensics is a multidisciplinary field dedicated to analyzing evidence from bio crime or bioterrorism or unintentional toxin release for imputation purpose. The ultimate goal of imputation is to identify the person who was involved in bio crime. Apart from microbiological tools, traditional forensic tools and techniques such as DNA analysis, fingerprint analysis, and tool mark examination will also be used to investigate a bio crime. If we compare forensic microbiological evidence with other forensic evidence, then there is nothing exceptionally unique. Recognition of crime

scene, preservation of crime scene, chain of custody, evidence collection, shipping of evidence, analysis of evidence, interpretation of result and presentation in court will be carried out in the same manner as it is done with other forensic evidence except that the evidence will be biohazardous. If there is no proper recognition of the crime scene, then it is quite impossible to identify the suspect or the nature of the incident, whether it was intentional or unintentional. Usually, bio crime and bioterrorism have been categorised in two parts, i.e. cases where crime has been committed in an undisguised manner or cases where crime has been committed covertly (Budowle et al. 2005a). Cases where no disguise is needed, one can place the pathogen in a public place or open space. In contrast, in covert cases unlikely occurrence of a particular disease will alarm the law enforcement agencies. In both cases, a partnership between public health and law enforcement is essential.

In order to plan better forensic investigation strategies, analysis of past cases can help in identifying critical problems and different approaches that should be adopted for active investigation. Some of the cases where epidemiological and molecular biology knowledge was used are Sverdlovsk anthrax case and the case of an HIV-infected dentist who might have infected patients with HIV (Jackson et al. 1998).

In the first case, sources of anthrax spores from an anthrax epidemic were explored. In April 1979 an outbreak of human anthrax occurred in Sverdlovsk, Russia. Although government officials attributed it to the consumption of contaminated meat, but western governments believed the cause to be a release of accidental spores from a nearby military research facility. At least two hundred patients died, and people affected from it lived within a narrow zone of approximately 4 km of south and east of military facility (Keim et al. 2011). Patient fatalities did not show any symptoms of skin anthrax; instead, they showed symptoms of pulmonary anthrax which ruled out the possibility of consumption of contaminated meat. Further investigation revealed that livestock from six different villages residing 50 km southeast of the facility also died due to anthrax. Tissue samples from 11 dead patients were examined. DNA extracted from tissue sample was analysed using the Polymerase Chain Reaction. B. anthracis toxin and capsular antigen genes required for pathogenicity were present in tissues from each of these victims.

In the second case, an attempt was made to determine the cause and source of HIV in patients that implicated a dentist based in Florida in 1980s (Keim et al. 2011). In the late 1980s, many people from Florida became HIV infected, although their lifestyle never put them in such kind of risk. Investigation indicated that HIV transmission occurred due to invasive dental care from a dentist with AIDS. The dentist was first identified as HIV positive in 1986. The actual mode of transmission of HIV was unknown, but the data implicated him as the source as the patients who got infected from HIV infection visited the same dentist. DNA sequences data from infected patient and dentist along with a local control group and outgroup were analysed. The analysis showed that HIV nucleotide sequences from several patients were closely related to the dentist.

4.4.1 Essential Components of Microbial Forensics Programs

- Proper identification and detection are essential for impeding bioterrorism. For effectively carrying out the attribution of an individual to a crime, robust analytical techniques should be developed, and proper implementation of those techniques needs to be done. Use of DNA based assays and analytical techniques of physics and chemistry can be used.
- Proper database and information will play an essential role in microbial forensics. Databases based on genomic sequences of bioagents and pathogenic agents need to be developed.
- Strain repository for the housing of pathogens and other related microorganisms should be developed. It will help in assay development and research work needed to be done for this field.
- Validation of new and existing methods should be done. It should not be limited to the procedures applied in the laboratory; tools used for interpretation of results should also be validated.
- Quality assurance guidelines should be established for microbial forensics laboratories (Budowle 2003).

To develop a proper foundation in the field of microbial forensics, a new group was initiated by Federal Bureau of Investigation known as Scientific Working on Microbial Genetics and Forensics (SWGMGF) on July 29, 2002 (LeBeau 2004). This group provides a common platform for interaction of scientists, academicians from different disciplines and various government agencies for guidelines development in the field of microbial forensics. SWGMGF vision is the development of infrastructure and tools for microbial forensics. The mission of SWGMGF is (Favero et al. 1968):

- defining criteria for development and validation of methods used in forensics for externalisation of biological toxins and microbial agents and,
- define the need for infrastructure development in forensics for active investigation.

As per SWGMGF, following requirements apply for a laboratory routinely involved in microbial forensics work (Budowle 2003):

- Documented procedure for each analytical technique used in the laboratory.
- The procedure should include a proper listing of equipment's, reagents, instructions used at each step, their limitations and literature references.
- The laboratory should have a policy whereby a deviation from an analytical procedure is documented and approved.

4.4.2 Steps in the Investigation of a Suspected Bioterrorism Case

The steps involved in identification of the microorganisms related to a crime scene are explained in Fig. 4.1. The salient aspects that need to be taken into consideration are explained in the following section.

- The suspected case of bio crime with an unusual outbreak of the disease in a place or area.
- Proper collection of samples from the crime scene, which should include every suspected material found at the crime scene. Proper labelling of evidence should be done by mentioning the time and date of collection.
- Samples collected from the crime scene should be considered as potentially biohazardous material, and processing of evidence should be done in a well-equipped laboratory.
- The microbial evidence is collected in the form of live cells, swabs of secretory toxins, DNA swabs, biological samples from the victim (Fluid, skin, hair), clothing and weapons of murder.
- It is imperative that the sample collection is robust, reliable and done sensitively and meticulously. This is because some samples can be very rare and difficult to catch (Bhatia et al. 2015).

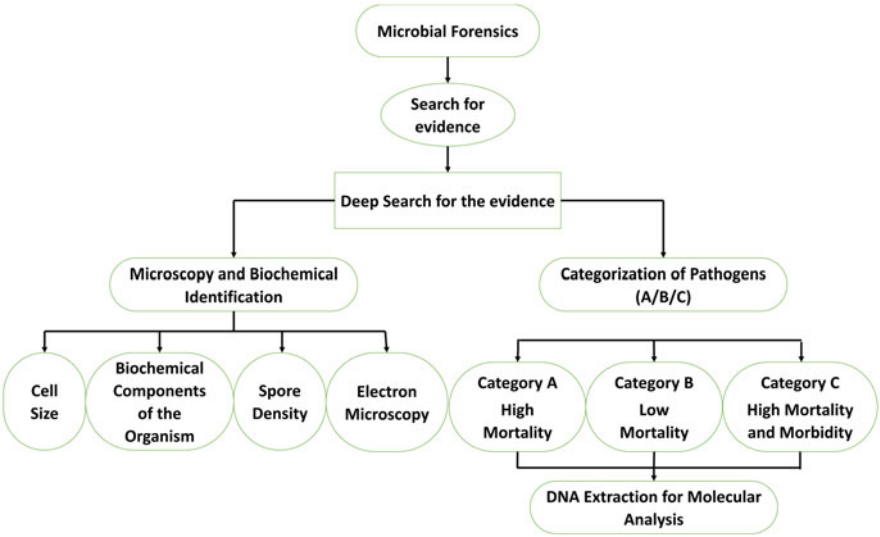

Fig. 4.1 Flow chart showing the steps involved in microbial forensic workflow

4.4.3 Methods Involved in Microbial Forensic Sampling

Correct sampling is quite essential for proper forensic evaluation and successful investigation. Microbial forensics samples can be collected by using three general approaches: bulk collection of an item, collection of a particular portion from an item or collection of liquid and swabbing of the surface (Rose et al. 2004). Bulk collection can be applied to only those items that can be removed easily from the area. Such items are packed appropriately and sent to a laboratory specifically designed to contain hazardous material or toxin. Samples can be collected from different surfaces by swabbing, wiping and vacuuming. It is essential to collect wet swabs, wet wipes and air filter samples throughout the room if bioweapon has been released in a closed confined space. It is also essential to collect the sample from the air vent and air handling units to identify the route of dispersal of the agents (Pattnaik and Sekhar 2008). Usually, sterile rayon swab dipped in phosphate buffer saline solution at pH 7.2 is used to swab a surface horizontally and vertically. From different studies, it has been found that the use of pre-moistened swabs on the porous and non-porous surface is more effective as compared to dry swabs. High-efficiency air particulate vacuum should also be used to collect a sample from the air (Rose et al. 2004). A similar methodology was followed during the investigation of *B. anthracis* contamination and anthrax inhalation case in Washington and New York (Keim et al. 2011). Adhesive tapes have shown better results as compared to pre-moistened swabs on flat porous, non-porous and non-absorbent surfaces (Frawley et al. 2008; Edmonds 2009). Swab collection has been proven more appropriate in case of a small sampling area with a high concentration of the agent, but it has limited value if we compare it with large sampling area. Sample collection form environment is essential for identifying the source of bioweapon agent to any particular geographical location. Apart from this, the sample should be collected from the affected person, his/her workplace and home by a trained medical professional. Use of vacuuming technique along with specialised equipment designed to collect environmental samples and prevent cross-contamination can be considered as an excellent collection method.

4.4.4 Techniques Used in Microbial Forensics

Microbial forensics relies on the DNA-based evidence as well. An important fact to be understood here is that the DNA of convict or victim may not always be available due to sample degradation. In such cases, the DNA of microorganisms associated with biological evidence can be used. The microbial forensics can use the metagenomic DNA from such evidences and analyse the DNA sequences for the purpose of comparing them with the known suspects. A typical workflow of this approach has been given in Fig. 4.2.

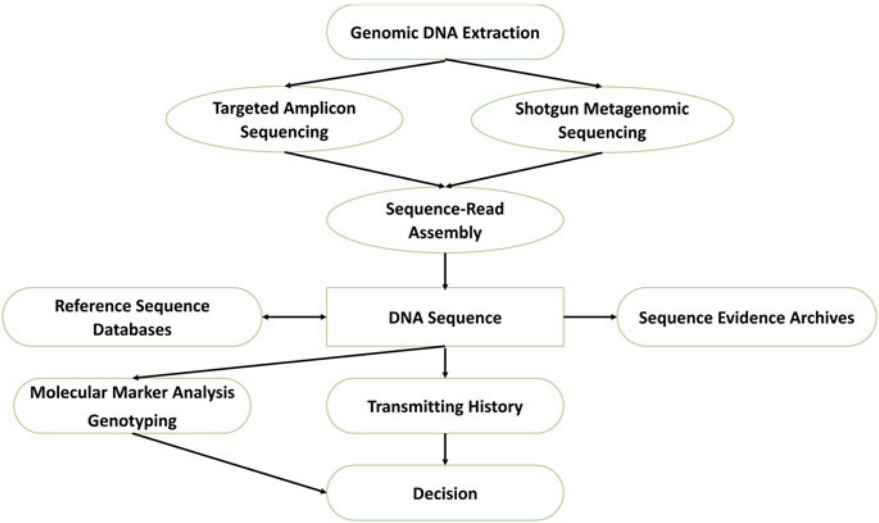

Fig. 4.2 Flow chart showing a typical workflow of molecular marker analysis of DNA and the metagenomic approach

4.4.4.1 MLST

It is a technique that could be used to compare the microorganisms based on the DNA sequence of a group of genes (Maiden et al. 1998). It is a useful method for genetic profiling of bacteria. In this technique, microorganisms are compared based on the "housekeeping" genes that are conserved sequences. Housekeeping genes are those genes that are constitutively expressed at stable levels in the organism as their expression is essential for the functioning of life. Along with the PCR, this technique can be used to amplify specific genetic sequences and can be compared. Reports show the application of MLST in the typing of many bacterial pathogens such as *Neisseria meninigitidis*, *Streptococcus pneumoniae*, among others. MLST data have been employed in epidemiological investigations of various scales and studies of the population biology, pathogenicity, and evolution of bacteria (Maiden 2006). MLST is especially preferred due to good reproducibility of results, the flexibility for analysis of various genes and the ease of analysis of sequencing data. However, the pathogenic strains can be fastidious and can have similar gene sequences. As a result, the sequence variability is less and the significance of results may vary with different sets of genes.

4.4.4.2 PCR-Based Genotyping

Multilocus Variable Number Tandem repeat analysis (MLVA) and Amplified Fragment Length Polymorphism (AFLP) are some of the PCR base typing techniques that are used. AFLP was first described in 1995 (Vos et al. 1995). It is a DNA profiling method combines the PCR and RFLP principles. Bacterial taxonomic research utilizes AFLP because of high-specificity and reproducibility leading to accurate differentiation between bacterial species (Janssen et al. 1996). There are

several conserved regions within the closely related pathogens. This is especially significant if the pathogens acquire similar genes through horizontal gene transfer. For such types of pathogen clusters, bi-allelic molecular markers may not be enough for profiling. For closely related groups of pathogens, the VNTR is the marker of choice. VNTRs show greater variability which allows their PCR amplification followed by sequence analysis and semi-quantitative analysis. The size of amplicons may vary based on the number of tandem repeats for the marker sequence (Keim et al. 2000). The VNTR analysis is easily automatable by using fluorescent primers instead of the conventional ones. With the advent of numerous fluorescent tags for DNA, the multiplexing of VNTR analysis is possible. Through multiplexing, a forensic scientist can study multiple loci in the same amplification reaction, hence the name Multilocus VNTR analysis (also known as MLVA) (Klevytska et al. 2001). This approach is successful in differentiating closely related isolates of organisms such as *Bacillus anthracis, Francisella tularensis* and *Yersinia pestis* (Farlow et al. 2001).

4.5 Conclusion

Microbial forensics is a branch of forensic sciences dealing with the microbiological evidence collected from a crime scene. These crime scenes could range from a homicide event up to an act persistent with bioterrorism and biological warfare. The illegitimate use or release of biological agents in the atmosphere or an ecosystem puts the native species at risk, including humans. This is a potential human health hazard and can wipe-out significant proportion of the populations. This makes the establishment of microbial forensics research and diagnostic facilities the need of the hour.

The biological weapons are invisible and are only traceable post-hoc. Microbial forensics allows ad-hoc screening of the various materials for potential microbiota used indiscriminately. Several examples are discussed in this chapter, which only shed light on the potential for microorganisms being used as weapons. The development of bioanalytical and molecular marker-based techniques has come a long way to enable forensic scientists to diagnose or detect the presence of pathogenic microorganisms. For the clinicians, there is need for sensitization and orientation on the management of the infective outbreaks that can potentially lead to pandemics. The need for development of rapid detection mechanisms through molecular-based methods, so that the relevance of microbial forensics is realized and the capacity for containing the outbreaks are built.

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