



ORIGINAL RESEARCH ARTICLE

Microbes in forensic medicine: a microbiologist perspectiveDeepak Narang^{1*}, Reena Kulshreshtra², Fatima Khan³, Jaideep Sur³, Amit Singh⁴, Vishal Kulkarni⁵, Veena Desai³, Abhishek Pandey⁶¹Department of Oral Medicine and Radiology, Dental College, Itaura, Azamgarh, Uttar Pradesh, India.²Department of Microbiology and Immunology, Rungta Dental College, Bhilai, Chhattisgarh, India.³Department of Oral Medicine and Radiology, Rungta Dental College, Bhilai, Chhattisgarh, India.⁴Mayo Institute of Medical Sciences, Barabanki, Uttar Pradesh, India.⁵Department of General Pathology, Government Medical College, Rajnandgaon, Chaatisgarh, India.⁶Faculty of Dentistry, Government Medical College, Akbarpur, Ambedkarnagar, Uttar Pradesh, India.

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Abstract: Microbial forensics is a naïve branch that involves multi-disciplinary approach to detection, tracing and evidencing the bio crime, with a predominant microbiological approach by studying evidence from inadvertent microorganism or its toxins released in bioterrorism act, biocrime, or for attribution purposes using advanced molecular techniques. Methods for identification and attribution of potential microbial weapons is challenging and forensic microbiological investigations involves crime scene investigation, chain of custody practices, evidence collection, handling and preservation, evidence shipping, analysis of evidence, interpretation of results, and court presentation. This field is emerging as a necessary for civil security rather than optional. In 21st century, pathogens are readily accessible, and technology is making their use as a weapon more feasible. As an preventive measures, it is important to strengthen microbial forensic capabilities. The sharing of data collected and validated will greatly improve the practice of microbial forensics. Forensic scientists worldwide should contribute to the field of microbial forensics and enhance its capabilities to aid in bringing perpetrators of these heinous attacks to justice. International and National collaborative approaches can be done by setting up a national and international reference laboratory, transparency of analysis.

Key words: Epidemiology; DNA; crime scene; biocrimes

Introduction

Microbial forensics is multidisciplinary branch that depends on the foundations of traditional forensic sciences, epidemiology, microbiology and molecular biology. According to Budowle, a new type of approach in which study of microorganism or its toxins using molecular techniques (Budowle B *et al.*, 2003). Microbial forensics practice is called molecular epidemiology from the branch of epidemiology and medical science. It mainly focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the etiology, distribution and prevention of disease within families and across populations. The characterization of DNA from crime scene samples able to use a set of only 10 to 17 microsatellite loci on the genome for most identifications (Budowle *et al.*, 2005). Epidemiologists attempt to determine the causes that are specific disease causing pathogens, toxins and their modes of transmission. Forensic science supports the investigations of hoaxes, which also cause disruption, economic loss, fear, and diversion of first responders (such as firemen, police, and paramedics), from normal duties and are also considered acts of terrorism.

A forensic investigation of a case, where the weapon was a pathogen or toxin, will attempt to determine the identity of the causal agent. For decades, epidemiologists have used general forensic practices to identify causative agents and the etiology of diseases for public health concerns. Molecular analyses of microbial agents from crime scenes are similar to those used in the human forensic DNA arena. Unique genetic identification of a microorganism may never be possible, because of the clonal nature of many microorganisms, less than optimal population and phylogenetic data, and in some instances limited historical and epidemiological information.

In bio crimes, one of the most significant threats to society is a serious disease outbreak by natural occurrence or intentional, resulting in harm or death, causing disruption, creating fear, and affecting economic wellbeing. Microbial pathogens or toxins can be used to commit acts of terror; they can be used as weapons in the commission of a crime. In biological warfare potential use of transmissible lethal agents to attack the targeted populations. The impact of the bioterrorism has seen many times in history, however it was seriously considered only after the anthrax attack

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in the United States in 2001. Anthrax letters attack of 2001 raised the awareness of our susceptibility and the potential importance of forensic analysis for attribution purposes of biological and non-biological samples used in a terrorism incident this leads the United States government sensing the potential of this area formalized “Microbial Forensics” as a branch [Budowle *et al.*, 2003]. As a preventive measure to stop the dangers of bio weaponry, BTWC (Biological and Toxin Weapons Convention) was established and now signed by more than 165 states. This treaty denies the use and stockpiling of biological weaponry or its use [The Biological and Toxins weapons convention website. <http://www.opbw.org/>. Accessed on June 15, 2016].

Microbes and its role

Microorganisms and its role has been identified in the fields of medicine, ecology, and fermentation science since long time, but they have been largely ignored by forensic scientists. However, this is likely to change as rapid advances in molecular sequencing and computational techniques are bringing about a revolution in the way we approach the study of these organisms (Segata *et al.*, 2012). Any microbiological agent can be successfully used as weapon, Because of the advantages using microbes or their toxins as weapons to commit crimes as they can be grown from a single organism or cells and unlike nuclear weapons without the need for sophisticated instruments and skilled man power (Priyabrata Pattnaik *et al.*, 2012), ease of access, the ability to amplify pathogens (by culture), and relatively low cost to generate the pathogen. Many pathogens can be found in nature and are endemic. Some organisms are fairly stable in the environment and can persist well after dissemination (such as *B. anthracis*) (Bruce Budowle *et al.*, 2005). Many of the pathogens used in past biocrimes are not as lethal as, for example, *Bacillus anthracis*, smallpox, or ebola, but they are readily accessible.

In microbial forensics, there is a multitude of species to characterize, being made even more complex by their biological and ecological dynamics. There is a number of potential bioweapons against human targets, considers naturally occurring infectious diseases account for 29 out of the 96 major causes of human mortality and morbidity and about 25% of global deaths (i.e., 14 million) per year. Of the 1,415 species known to be pathogenic to humans, 217 are viruses and prions, 538 are bacteria and rickettsia, 61% are zoonotic (i.e., transmitted between humans and animals), 33% are transmissible among humans, and only 3% have humans as their main reservoir (Taylor LH, *et al.*, 2001). Thus, there is an abundance of potential pathogens that can be exploited.

In a case investigation of bite mark inflicted case, characterization of the microbiota of the oral cavity reveals the initial colonizers of the teeth that exhibits considerable genotypic diversity in the predominant organism (Nyvad B, Kilian 1987; Pearce *et al.*, 1995; Hohwy 2001). Humans harbor multiple strains of the same Streptococcus species with many strains seemingly unique to individuals [Fitzsimmons *et al.*, 1996]; Wisplinghoff *et al.*, 1999; Rudney JD and Larson CJ *et al.*, 1994]. This intra species diversity provides the premise that oral isolated strains from a bite mark inflicted on human skin may be genotypically matched, with a high degree of assurance, to those from the teeth responsible [Borgula *et al.*, 2003; Rahimi 2005]. These observations were reiterated in a third study (Hsu *et al.*, 2012) that circumvented the need for prior culturing by amplifying bacterial DNA directly from teeth and experimental bite marks. In that study, streptococcal DNA, amplified with primers specific for hypervariable region 9 of streptococcal 16S rRNA gene, was resolved by denaturing gradient gel electrophoresis (DGGE), and a comparison of the amplicon profiles from the bite marks and teeth matched most bite marks to the teeth responsible. However, there was a concomitant risk of false positives with the sole use of this relatively conserved locus [Hsu *et al.*, 2012].

The important stages involving in the investigation begins with DNA isolation in which extraction and purification of bacterial DNA from the skin, bite mark and teeth samples was achieved followed by PCR Amplification for that streptococcus-specific oligonucleotide primers for the amplification of approximately 245 base pair (bp) fragments of the 16S rRNA gene; 16S–23S rRNA intergenic spacer region (ITS); endoribonuclease P (*rnpB*); and RNA polymerase betasubunit (*rpoB*) loci. From a forensic standpoint, assurance that there is temporal stability of oral streptococcal populations is crucial. Genetic analyses reveal that oral streptococcal populations are dynamic with species numbers and proportions fluctuating over time (Hohwy *et al.*, 2001; Bek-Thomsen *et al.*, 2008]. The mechanisms underlying these changes are not fully understood; however, the dominant strains of streptococci are generally retained over longer periods (Rahimi *et al.*, 2005). Rahimi *et al.*, 2005, found that between 20–78% of bacterial genotypes were recovered from the same teeth 12months later. The likelihood of matching bite mark sequence data to that of a suspected assailant will be increased by prompt sampling. The comparison of highly discriminatory regions of oral streptococcal DNA recovered from bite marks and teeth is capable of unequivocally matching a bite mark to the teeth responsible and may provide valuable information to corroborate other evidence in cases where the perpetrators DNA cannot be recovered.

Role of microbes in decomposition processes

The forensic potential of microorganisms is increasing due to the advancement in molecular sciences and genomics. Microbes are rarely present in cerebrospinal fluids and blood of a healthy individual, if a person dies these microorganisms can proliferate entire body and starts to break down (Morris *et al.*, 2006). From a medico-legalistic perspective, the agonal period is difficult to define (Mason, 2010). After death, the speed of microbes move through the body is affected by the environmental conditions and whether or not the dead person suffered wounds that facilitated their entry. If the body is frozen immediately after death, then, obviously, there is no movement of microorganisms and decomposition does not take place. Morgue refrigerators typically operate at 1°C to +4°C and therefore delay but do not prevent the decay process. However, provided the body is placed in cold storage shortly after death, this is usually sufficient to reduce the likelihood of microbes invading the body for at least 24 hours (Morris, 2007a). This is an important consideration when there are concerns about whether organisms recovered at post-mortem contributed to the person's death. As the environmental temperature increases, the microorganisms present upon and within a dead body will replicate faster and as a consequence the pH of the blood becomes acidic and the fluids and tissues become anaerobic. High humidity can also facilitate the growth of microorganisms and therefore decay is faster in the humid tropics than it is in cool temperate climates. The Gram-positive bacillus *Clostridium perfringens* can come to dominate the microbial community since it has a doubling-time of only 8 minutes under optimal conditions (Spicer, W.J. 2000), which is faster than most other bacteria typically found on a dead body. Bacterial putrefaction results in the loss of body tissues (and hence, of potential evidence) and generates gases such as carbon dioxide, hydrogen sulphide, and methane, that are responsible for the bloat stage of decomposition. In addition, phenethylamine, tryptamine and other amines are generated (Stevens, H.M. 1984). Bloat often ruptures the skin surface and this allows the entrance of oxygen thereby facilitating the return of aerobic decay processes. Volatile chemicals such as mercaptans released by bacteria attract invertebrate and vertebrate detritivores that then feed on the corpse and contribute to the loss of tissues. To date, relatively few studies have been done on the microbiome of dead bodies in a forensic context. Consequently, although it is to be expected that the microbial species profile will change as the body undergoes the various stages of decay it is not yet possible to use this as an indicator of the time since death. Similarly, a dead body can be expected to alter the microbial abundance and profile of the soil or other matrix on which it is found in a time-dependent manner but there is as

yet limited information on this (Howard & Watson 2010).

In decay process, microbes degrade certain drugs and also generate metabolites that can be mistaken as indicators of pre-mortem drug consumption. For example, nitrobenzodiazepines such as Clonazepam and Nitrazepam, are quickly broken down by bacteria to amino compounds and may be difficult to find in the blood even when the victim has died of an overdose (Robertson & Drummer, 1995; Drummer, 2007). By contrast, morphine can be detected in buried bodies up to 8 years after death (Skopp, 2004; 2010) although during this time morphine-3-glucuronide is converted to free morphine by bacteria (Carroll *et al.*, 2000).

People or bodies are usually identified from their DNA and morphological characteristics. In the case of trace evidence, such as body fluids, human DNA is also the best identifier, whilst fingerprints and other contact evidences were analysed using physical characteristics. However, it is not always possible to extract a full DNA profile and fingerprints are often smudged and incomplete. Studies on the human microbiome indicate that not only are there major differences in the microbial composition within regions of the body but that there appear to be consistent differences between individuals (Costello *et al.*, 2009).

Microorganisms can contribute to forensic investigations in a variety of ways from the identification of body fluids to tracing the source of a bioterrorist incident. Therefore, whilst forensic microbiology offers enormous potential to a wide variety of criminal investigations, there is a need for a great deal of basic research before this can be realized.

Role of Microbiologists

Forensic microbiology used in conjunction with forensic anthropology can be used to help trace individuals to specific areas. Forensic microbiological investigations are essentially the same as any other forensic investigation regarding processing. They involve crime scene(s) investigation, chain of custody practices, evidence collection, handling and preservation, evidence shipping, analysis of evidence, interpretation of results, and court presentation.

Microbiologists study genes, pathogens and contagions in a never-ending fight to keep people safe and to track those who bring them harm. Forensic microbiologists burrow deep below the visible world to find biological markers hidden in the DNA of all living matter. These markers, known as "microsatellites," identify patterns in DNA that help determine everything from a person's individual identity to the direct link

between two seemingly dissimilar outbreaks of food poisoning in different parts of the country.

The main goals of microbial forensics is to identify and prioritize biological threats, identify the vulnerable population, create an information database and develop protocols for identification which includes determining unique genetic signatures, protein signatures, develop programs for ensuring the validity of results and constantly update based on existing literature [Microbial forensics: A new forensic discipline, Sharad Jain, Ashish Kumar, 2005; Defining a New Forensic Discipline: Microbial Forensics Bruce Budowle Laboratory Division, Federal Bureau of Investigation, Washington, DC, U.S.A. March 2003. www.promega.com. Accessed on Dec 20, 2011]. Forensic scientists often analyze materials for comparison purposes to eliminate them as potential sources of the evidence. However, chemical characterization of a microorganism or its matrix may assist an investigation by providing information regarding the processes used to grow the pathogenic agent.

Sample collection

Forensics role begins with a suspected case with an unusual presentation. The steps involved in the investigation are essentially the same as investigation of a natural outbreak. However, they are more demanding than the routine diagnostic or epidemiological assays (Ronald, 2004). The sample collection is of utmost importance. The samples to be collected include every material found in the scene which is labelled with time and site of collection. The name of the person who has collected the sample should also be mentioned. The code of practice should be the same irrespective of the type of the sample from a community or individual.

Microbiological evidence could include; viable samples of the microbial agent, protein toxins, nucleic acids, clinical specimens from victims, laboratory equipment, dissemination devices and their contents, environmental samples, contaminated clothing, or trace evidence specific to the process that produced and / or weaponized the biological agent. On the forensic front the method of collection should be sensitive, reliable and robust to clinch the presence of possible organism or the toxin [National science and technology council. National strategy to support research in microbial forensics attribution investigations and national security. <http://www.whitehouse.gov/files/documents/ostp/NSTC%20Reports/National%20MicroForensics%20R&DStrategy%202009%20UNLIMITED%20DISTRIBUTION.pdf>. Accessed on June 24, 2016]. Timely environmental sampling is of immense value as it may be rapidly destroyed and the evidence of intentional spread may be lost.

Each sample should be considered potentially hazardous and processed only in a well-equipped laboratory, or ideally sent to a reference laboratory equipped with stringent bio safety levels (Ronald M. Atlas. 2004).

Technology in Analysis

The methods used in analyzing samples are classical microbiology techniques, serology and nucleic acid based techniques. There are more techniques are being investigated to molecular signatures or molecular markers, which are reliable and quantifiable (Pattnaik, *et al.*, 2008). The first steps involve routine microbiological methods that the organism may be totally unknown or unidentifiable by traditional microbiology practices. Once the possible organism is identified, all possible analysis such as genetic fingerprint, unique DNA sequences protein signatures were investigated to establish and strengthen the data statistically.

Microbial forensics analyse and characterize forensic evidence with advanced molecular techniques like microarray analysis and DNA fingerprinting etc. Nucleic acid amplification and molecular epidemiological techniques are essential tools in clinical microbiology for identifying pathogens and in out-break investigations. DNA/RNA typing figure prominently in the cadre of analytical tools for microbial identification and characterization purposes. (Budowle B, *et al.*, 2005) Various Nucleic acid based typing technologies include: PCR, real-time PCR, MLST (multilocus sequence typing), MLVA (multi-locus VNTR analysis), FISH (fluorescent in situ hybridization), whole genome sequencing and microarrays (Budowle B, *et al.*, 2005). Additional new methodologies like Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF), Gas Chromatography-Mass Spectroscopy (GC-MS), Liquid Chromatography-Mass Spectroscopy (LC-MS) are also well established in resolving minor difference in proteins. These methodologies can be used to detect and trace back the spread of microorganisms in the context of a crime and the procedures exemplify how molecular biology tools may aid in an investigative process.

Recently molecular biology has resulted in the development of numerous DNA-based methods for discrimination among bacterial strains for e.g. The use of Simple Sequence Repeats (SSR) for bacterial typing, selected MNR loci were analyzed for variation among strains belonging to bacterial species. SSRs are a class of short DNA sequence motifs that are tandemly repeated at a specific locus. Simple Sequence Repeats (SSRs) also termed VNTR (Variable Number of Tandem Repeats). Mono Nucleotides Repeats (MNRs), a subgroup of SSRs is the mononucleotide repeats

(MNR) (Gur-Arie R, *et al.*, 2000; Keim P, *et al.*, 1999; Keim P, *et al.*, 2000). Species identification is mainly by biochemical characterization and strain identification is primarily based on serology, PFGE and PCR-based methods; Repetitive extragenic palindrome (REP-PCR) (Olive MD, *et al.*, 1999; Van Belkum A, 2003; Weidemann M, 2002) DNA-sequence based method, for bacterial strain typing (Clarke SC. 2002; Maiden MC, *et al.*, 1998). DNA sequencing provides far more variation per locus than any other method currently used for bacterial strain typing, and it provides a uniform platform for comparison between different laboratories and for database storage.

Validation

Microbial forensic scientists have very good command over technologies they are using for proper validation so that the methods used were understood and interpretation of results that were carried out within the limitations of the assays. There are three types of validations that are preliminary, developmental, and internal.

Preliminary validation is an early evaluation of a method that will be used to investigate a biocrime where the method used have not been through external and internal validation but are deemed necessary to support an investigation event. (Budowle B, *et al.*, 2008) One approach to acceptably achieve a preliminary validation is to convene a panel of experts, to assess the utility of the rapidly developed method, and to define the limits of interpretation and conclusions. Such an approach has been employed in the field of human DNA forensics for victim identifications in the disasters at the twin towers of the World Trade Centre in New York on September 11, 2001. (Budowle B, *et al.*, 2005)

Developmental validation is the acquisition of test data and the determination of conditions and limitations of a newly developed methodology for use on samples. (Budowle B, *et al.*, 2007) Once a method has been developed and initially validated, it may be transferred to an operational laboratory for implementation.

Internal validation is an accumulation of test data within the laboratory to demonstrate that established method performs within determined limits in the laboratory. These two types of validation are crucial for addressing the reliability and robustness of any method routinely implemented in the laboratory. (Budowle B, *et al.*, 2007)

All components of a microbial forensics investigation need to be validated from methods for collection, sampling, preservation and handling in order to identify the agent. Establishing criteria

and requirements for validation, and compiling a list of all validated protocols in use (e.g., for sampling, DNA extraction and isolation, and sequencing) would help ensure the quality of microbial forensic evidence.

Bioinformatics in forensic medicine

As new sequencing methods generate huge amounts of data, needs are growing for novel ways to handle and analyze this information. Advances in bioinformatics, an interdisciplinary field that develops and improves on methods for storing, retrieving, organizing, and analyzing biological data needed to help meet these needs. Bioinformatics also needs to be made understandable and user-friendly to laboratory users, first responders, the public, and policy makers.

Data Sharing

Data sharing from biological information on gene sequences to software, protocols, and standard methods for microbial forensics procedures is critical. Sharing such data has the potential to promote international collaboration and cooperation among scientists, and, more importantly, inspire innovation. Establishing a comprehensive archive of reference materials, including organisms, nucleic acids, and sequence information could facilitate the development of standardized nomenclature and techniques and provide references for genomic comparisons. Such a facility should take advantage of existing models, such as the American Type Culture Collection and the World Data Centre for Microorganisms.

Conclusion

Microbial forensics is a naïve branch that involves multi-disciplinary approach for the detection, tracing and evidencing, with a predominant microbiological approach. This field is emerging as a necessity for civil security rather than luxury. In 21st century, pathogens are readily accessible, and technology is making their use as a weapon more feasible. As a preventive measure, it is important to strengthen microbial forensic capabilities. An effective program will require development and validation of all aspects of the forensic investigative process, from sampling to interpretation of results. There is a need to rely on other existing and emerging capabilities beyond the traditional forensic laboratory and its practitioners. The sharing of data collected and validated will greatly improve the practice of microbial forensics. Forensic scientists worldwide should contribute to the field of microbial forensics and enhance its capabilities to aid in bringing perpetrators of these heinous attacks to justice. International and National collaborative approaches can be done by setting up a national and international reference laboratory, transparency of analysis.

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