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Review article

Microbial Forensics- Past, Present and Future

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ABSTRACT

Bio terrorism in both crude and refined forms have been seen throughout the ages even from Biblical times. Terrorist or criminal use of pathogenic organisms and their toxins have been of great concern ever since the Anthrax attacks in 2001 in the United States. A relatively naive field in many nations world over, Microbial Forensics should play a lead role in sample handling, tackling, configuring, prioritizing and validating bio crimes. The objectives of this review on Microbial forensics is to look back into the subtle events of the past, assess the shocking incidents of our present times and propose prospects to systematically deal with this emerging menace in the future and secure the nations with a special reference to India from the disastrous consequences of bio weapons.

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1. Introduction

Microbial forensics may be defined as a scientific discipline bridging microbiology and forensic science dedicated to track and analyse bio crime. Microbial forensics includes a vast scope of forensic science clubbed with microbiology, which includes but not limited to analysis of microbes or their toxins and materials used to prepare, store and deliver the toxin or pathogen. This new branch is essential for the modern world where the technology possessed by the criminal or a terrorist could be disastrous. The impact of the attack has been seen multiple times in history, however it was seriously considered only after the anthrax attack in the United States in 2001. The United States government sensing the potential of this area formalised "Microbial Forensics" as a branch [1]. The reason for such an attack could be political or personal, inflicting threat locally or globally. It is predictable that many private illegal organisations could possess biological weapon (s). Biological warfare makes use of transmissible, potentially lethal agents to attack the target populations. The motivation is to have a high impact on the target, which because of its self sustainability can inflict high damage at local levels. As can be made out, this approach is potentially dangerous as it implants new organisms into the environment that can have devastating consequences.

2. Past history and recent issues

Biological warfare is not a new age weapon, but a well documented approach though less scientifically advanced. The Bible mentions of God's demand for the Israelite slaves to be freed by Pharaoh. When Pharaoh did not comply, God sent upon Egypt ten deadly plagues [2]. Other early examples for these include use of poisonous snakes [3], dead animals to contaminate water sources and throwing of plague infected bodies (summarized by Friedrich) [4].

Tricothecenes, a type of Mycotoxin, is supposed to have been used in the famous yellow rain incident [5] which still is a subject of debate with no conclusive established proof, though it's thought to be implicated at least in some. A case was documented in the US where a gastroenterologist was convicted of attempting a second-degree murder by injecting his former girlfriend with blood or blood-products obtained from an HIV-1 (Human immunodeficiency virus-1) infected patient under his care [6]. Other recent well publicized bio-crimes include use of "ricin" in Europe for the assassination of a Bulgarian exile, a laboratory worker intentionally infecting co-workers in Texas with *Shigella dysenteriae* [7] and use of *Salmonella Typhimurium* in Oregon by contaminating salad bars in local restaurants which was politically motivated [8]. The most well known attack of bioterrorism in the present century is the use of anthrax spores in New York in October 2001 [9].

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

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treaty denies the use and stockpiling of biological weaponry or its use [10].

3.The Bioweapons concept

Microorganisms are potential weapons because they can be grown from a single organism or cells and unlike nuclear weapons, can be produced in a small establishment with low capital investment and without the need for sophisticated instruments and skilled man power [11]. Though there are many methods to create biological weapons the common option would be to release microorganism that is potentially pathogenic or may be attenuated to more pathogenic forms and then released into the community which makes it most lethal than any other weapon. Any microbiological agent can be successfully used as weaponry, of which the primary focus will be an agent which is more environment resistant and pathogenic. With the use of genetic engineering, even an environmental commensal may be equipped with an effective toxin producing gene. This would make it environmentally stable and a highly effective weapon.

A list of the possible species as a bio threat is beyond the scope of writing as any organism may be attenuated and used as the trigger. For a list of high alert organisms, readers are referred to Friedrich et.al [4] and the Centre For Disease Control (CDC) website [12]. The detection method must include a protocol that can be sensitive, specific and rapid. Molecular diagnosis favours the maximum in such a setting. It's applicability in a developing country can however be challenging, owing to resources. Moreover many organisms involved in the crime may be totally unknown or the DNA sequence partially known which makes it difficult to develop a molecular assay.

4.Tackling Bio-crime

The main goals of microbial forensics would be 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 [13, 14].

Identifying and prioritizing the target is more difficult than commonly thought. The organism that needs to be prioritized may be totally unknown or the organism may not be a human pathogen, but rather a plant pathogen which can inflict high economic damage [15, 16]. Identification of vulnerable population would be equally challenging. Creating an information database would help in guiding towards the results, however on the contrary it can also help the criminal in identifying those organisms which are not categorized and thus make way for the proliferation of an organism more difficult to identify. Authenticity of use of such a database should be clear for the same reasons. The database that is used will involve input from various classical fields inclusive of microbiology, genomics, forensic methods, chemistry and pure science [17]. A potential list of threats should be readily available to all concerned. This should be referred as priority targets.

5.Quality Control & Validation

The quality assurance and quality control program is an inevitable part of any laboratory analysis and microbial forensics is not an exception. Developing a protocol for identification may make use of routine diagnostic policies, for which quality guidelines exist. In addition techniques that have not undergone validation may also have to be used, especially when the organism is unknown or very rarely encountered which is currently not recommended by any guiding documentations. Such results may not be highly reliable but can create clues for judging the possible organism. This is of special concern as the organism dealing with may not be much known to the scientific world. Scientific Working Group on Microbial Genetics and Forensics (SWGMGF) establishes and sustains guidelines and / or standards for quality systems identifying processes and procedures, define criteria for knowledge systems, and most importantly serve as an experienced resource on issues as they arise [14]. The SWGMGF defines the guidelines and updates it as and when required. The development of these guidelines helps the laboratory to perform various forensic analyses and host the results as valid and true to the best of scientific knowledge available to that day.

Additional stringent rules are required [17] compared to routine surveys as the issues involve legal matters and data will be relied upon heavily. An erroneous quality management or lack of quality control may mislead the final conclusion. The chain of custody should be impeccable to obtain perfect results, especially the biologic evidences obtained in this context. A standard operating procedure (SOP) may not be available always and often guidance from other institutions may be required and opinions considered. In addition constructing a validation plan and its execution will help the cause [17, 18]. The development of rules should be based on standards of human DNA typing, clinical laboratories standards and International Standards Organization.

6.From sampling to diagnostics

The role begins with a suspected case with an unusual presentation or in a place where distribution of disease is unusual. In case of a bio-crime, generally the laboratory that obtains the sample as routine assay is the one to first raise a suspicion. If a strong suspicion is invoked it should be communicated to an investigative body or national reference centre, especially when a strain that looks genetically engineered or sample analysis shows multi-strains of possible aetiology [19]. For more indicator of suspicion refer Treadwell TA et.al [19]. 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 [20].

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 [21]. Timely environmental sampling is of immense value as it may be rapidly destroyed and the evidence of intentional spread may be lost [17]. 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 [20].

7. Identification approach

The diagnostic methodologies used in the analysis are Classical microbiology techniques, serology and nucleic acid based techniques. Currently, more emphasis is being given to molecular signatures or molecular markers, which are reliable and quantifiable [22]. The method of analysis could be very challenging especially in an environmental sample [17]. The first steps would be to follow routine microbiological methods keeping alive the fact that the organism may be totally unknown or unidentifiable by traditional microbiology practices. Once the possible organism is in limelight all possible analysis such as genetic fingerprint, unique DNA sequences, protein signatures may be required to establish and strengthen the data at least statistically. New methodologies like Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF), Matrix Assisted Laser Desorption Ionization-Time of Flight- Mass Spectrometer (MALDI-TOF-MS), Gas Chromatography- Mass Spectroscopy (GC-MS), Liquid Chromatography-Mass Spectroscopy (LC-MS) are well established in resolving minor difference in proteins [22]. These may help in identifying the unique protein signatures. Restriction Fragment Length Polymorphism (RFLP) may help in identifying genetic signatures and polymorphism [23]. Given the probability that any method will have at least a minor percentage of inherent error the procedure may have to be repeated to ascertain the test results as valid. This would especially be the case if prior quality tests are not available for the particular method used. Tracing the source may require in addition to the said methods, an isotope analysis as outlined by Kreuzer-Martin et.al [24, 25]. Using genetic signatures, whole genome sequencing [26] and environmental analysis it should be possible to identify the infections as natural or customised.

Similarities between human forensic and microbial forensic DNA analysis exist, such as use of population databases, qualitative conclusions of test results, and the application of quality assurance / quality control practices. The differences would include the database size [27], contents and the protocol for analysis. This information can direct law enforcement officials (like a central investigating agency of that country) to expertise on specific threat agents. To achieve this goal, a national database (s) of pathogens, pathogen profiles and individuals authorized to have access to these pathogens and

their data must be established [9]. Unlike an epidemiological survey, here the samples should be maintained till the clearance of the report, that it is a natural outbreak by a designated authority based on scientific observations, or hold it as an evidence for judiciary purpose. The strain may be required to be maintained by the judicial system or may be destroyed as required by the system.

8. Legal Issues in Microbial Forensics

Microbial forensics as already discussed, in right hands is an elegant tool to tackle bio-crimes. However this comes at a cost. To elaborate, consider A hypothetical case where an organism which sustains the possibility of its use is detected at a crime scene is isolated and characterized. By using well established scientific assays, the origin can be traced to a laboratory or person. Once the person matching the profiles is found he/she will be summoned. The judicial system has to prove his/her willing biocrime act. The first hurdle here is to prove his/her motive. The second is that the organism has come from him/her. This might be at times impossible to prove owing to the fact that same genotype of organism exists in nature and he/she may not be the only source harboring it [28]. In this hypothetical case, all the efforts to nail down the criminal may be ineffective unless a strong statistical evidence is created with unique signatures from the organism and the entire purpose of doing it may be jeopardized.

9. Scope of microbial forensics in India

Although the United States is currently believed to possess technically sound and most advanced capability for microbial forensics, a number of other countries are engaged in this field and pursuing research and development on microbial forensics and developing capabilities to analyse evidence from a possible bioterrorist incident [29]. In context to the Indian scenario, the potential of microbial forensics is incredibly vast. As a country which is commonly threatened by terror attacks, there is no doubt that biological weapons will be made use of by illegal organisations. Establishment of a national organisation which integrates specialities from various fields of science will prove to be beneficial despite the cost that will be incurred in creating and maintaining such a team.

Three components will be absolutely essential to establish a fully functional National Microbial Forensic Laboratory. The first would be a knowledge centre composed of databases on genomics, microbiology, forensics methods, SOP, evidence assays such as fingerprinting, bioinformatics and standardised tools. The second component will be maintenance of a strong partnerships between the existing government, the laboratory in charges, scientists and investigating agents. The third component will be quality control and validation of newer assays [30].

To the best of the authors knowledge, there is no documented case of biological materials used by individuals in India, to intentionally cause harm to others. This may be interpreted as no case occurring or as lack of forensic microbiology work up. The case of Anthrax spores (Amerithrax) was under study for many years [31]. This is partially because United States was not prepared to face it, though a strong

political and scientific support was able to quarantine it quickly. This highlights that the lack of preparedness to this menace may result in high cost and damage to the country financially and mentally. In any case, in future Microbial Forensics should have a positive impact on the population of the country in keeping the civilians safe. The knowledge of the same will help in quarantining the problem if at all the need arises, which would be difficult if a Microbial Forensic department doesn't exist. So it is safe to conclusively say that Microbial forensics is an essential requirement in India.

10. Summary

In a nutshell, Microbial forensics is a naïve branch that involves multi-disciplinary approach to detection, tracing and evidencing the bio crime, with a predominant microbiological approach. This field is emerging as a requirement for civil security rather than luxury. A national and international collaborative approach against the menace of bioterrorism can be done by setting up a national and international reference laboratory, transparency of analysis and strict action against all bio crime perpetrators. Considering all the scientific facts already discussed, "Microbial Forensics" should be an ideal requirement in India.

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