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Microbiology with An Emphasis on Forensic Applications

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ABSTRACT *The aim of this manuscript is to provide the concepts of microbiology with an emphasis on forensic applications. Microbial forensics has been closely associated with the process of attribution, or identifying the perpetrator of a biological attack for purposes of criminal prosecution. Pathogens and toxins can be converted to bio weapons and used to commit bioterrorism and bio crime. Microbial forensics is focused on characterization of evidence from a bioterrorism act, bio crime, hoax, or an inadvertent release. The forensic investigation will attempt to determine the etiology, where did the specific organism come from, who was responsible and identity of the causal agent. If a laboratory scientist suspects bio terror or bio crime, or other legal case, law enforcement agents must be notified and diagnostic samples preserved.*

Introduction:
Microbial forensic analysis defined as "the detection of reliably measured molecular variations between microbial strains and their use to infer the origin, relationships, or transmission route of a particular isolate" [1]. Forensic microbiology or microbial forensics examines the outbreak of a disease, looks for the pathogen that is responsible, and traces it to the origin of the outbreak. A number of these pathogens can affect both humans and animals, can contaminate the environment for decades, or may establish new enzootic foci. The forensic investigation will attempt to determine the etiology, where did the specific organism come from, who was responsible and identity of the causal agent.

Comparative evaluation of specific gene targets having more number of synonymous mutations has been explored for molecular typing and tracing the source of pathogens in disease outbreaks. [2]. The application of microbial forensics is to assist in resolving bio crimes, with a focus on research and education needs to facilitate the use of microbial forensics in criminal investigations and the subsequent prosecution of bio crimes, including acts of bioterrorism. The accuracy and precision will depend on the typing method, expected mutation rates, and other characteristics of the organism. Techniques for forensic microbiology can be very similar to those being used for phylogenetic and epidemiological investigations e.g. for food-borne outbreaks.

Many of the principles of forensic microbiology build off of the work of molecular epidemiology while making it admissible in court. High quality assurance and quality control standards for microbial forensics will ensure highly reliable results that will stand up in the court of law. Mutations and other factors can lead to differences in the DNA of evidential samples. Forensic microbiologists must prove that such differences are natural and do not indicate that the two samples are from different sources. Often this is done by examining one or a limited number of genetic loci.

However, the recent demonstration that the entire genome of an organism can be replaced with that of another [3] means that it may be possible to create designer pathogens by fusing genome parts of several different organisms, possibly with some totally synthetic DNA. As a result, the forensic microbiology investigator must be able to survey the causative agent's entire genome to identify the sources of each of its parts. MLST involves determining the nucleotide sequence of a few genes conserved within the taxon being tested [4], MLVA, for example [5], RAPD and AFLP methods [6], SNaPshot [7], Microarray applications [8] Bodrossy& Sessitsch, 2004]; [9-10] SNPs can also be surveyed by microarray methods [11].

Development of Forensic Microbiology:
The use of bioweapon, bioterrorism and bio warfare throughout history has been well-documented [12] back to 6th Century B.C. when Assyrians poisoned the wells of their enemies with rye ergot. Forensic Microbiology came into its own due to the anthrax scare in 2001 [13]; the most well-known attack of bioterrorism in the present century is that by the anthrax spores in New York in Oct.2001 [14] but also dealt with the intentional spread of HIV by carriers and outbreaks of hospital-acquired or food born disease [15]. Due to the anthrax letters, the FBI set up the Scientific Working Group on Microbial Genetics and Forensics (SWGMGF) to lay the foundations of microbial forensics as a field. The brief historical review seen below in **table 1:**

Table 1: The brief historical review

Time Period	Infection Causative Agents	Effects of Pathological Strains
430-426 BC	Plague of Athens	Sparta's winning the Peloponnesian War
14th century	Plague	The Black Death in Europe
1754-1767	Smallpox virus	During French and Indian war
Mid-1800s	Inhalational anthrax	Woolsorters' disease in England and Ragpickers' disease in Germany/ Austria
Early 1900s	Inhalational anthrax	Human cases in United States
During the 1st World War	Anthrax and Glanders	Germany in an effort to destroy animals
During the 2nd World War	5 million anthrax-laden linseed-oil 'cattle cakes'	In 'Operation Vegetarian' the United Kingdom (UK)
1932	Plague	Infected 1,000—2,22,000 persons

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1940	Yersinia pestus	The Chinese dropped ceramic containers holding plague infested fleas on Manchuria
1976	Inhalational anthrax	USA
1978 -- 1980	Anthrax outbreak	Zimbabwe
1979	B. anthraces spores	The largest outbreak of inhalational anthrax in the 20th century in Sverdlovsk, 94 people were infected, 68 died
1984	salmonella	Outbreak affected at least 751 people and 45 of them were hospitalised in the Dalles, Oregon
Sept. and Oct. 1984	Salmonella gastroenteritis	751 persons infected in The Dalles, Oregon
1992	Cyanide	Poisoning water supplies with cyanide in Turkey
1995	Sarin gas	killing 12 peoples in Tokyo subway
August 1997	Rabbit calicivirus disease (RCD)	Diagnosed by the Animal Health Laboratory in Wallaceville, New Zealand
1950's and 1960's	Francisella tularensis	United States during the US offensive bio warfare program
1999	West Nile virus (WNV)	Mortality in wild bird species, illness& mortality in humans New York City, North America
May 2000	E. coli O157: H7 and C. jejuni	An outbreak of waterborne illness, 2300 cases of illness and 7 deaths in Walkerton, Canada
2001	Anthrax letters	22 cases including 5 deaths in the USA
Nov.2002--July 2003	SARS corona virus (SARS-CoV)	Severe Acute Respiratory Syndrome (SARS) an outbreak of SARS in Hong Kong 8,273 cases and 775 deaths worldwide
2004	Highly pathogenic H5N1 influenza	Thai eagles were smuggled in Belgium
2004	Outbreak of Glanders in horses	Office International des Epizooties by the United Arab Emirates
Oct. 2004	Highly pathogenic H5N1 influenza virus	25 persons with oseltamivir prophylaxis and destruction of over 650 birds in quarantine in Thailand

Biological agents used as bio-weapons in different bio-crimes:
Biological agents include bacteria, viruses, fungi, other microorganisms and their associated toxins. They have the ability to adversely affect human health in a variety of ways, ranging from relatively mild, allergic reactions to serious medical conditions, even death. These organisms are widespread in the natural environment; they are found in water, soil, plants, and animals. Because many microbes reproduce rapidly and require minimal resources for survival, they are a potential danger in a wide variety of occupational settings.

The most dangerous agents [16] and example of diseases caused by them are seen below in table 2:
Table 2: The most dangerous agents

Causative Agent	Disease Name
Variola major virus	Smallpox
Bacillus anthraces	Anthrax
Yersinia pestis	Plague
Clostridium botulinum toxin	Botulism
Francisella tularensis	Tularemia
V Viruses	Viral hemorrhagic fever
SARS corona virus	Severe Acute Respiratory Syndrome
Hepatitis B	HBV
Hepatitis C	HCV
Human Immunodeficiency Virus	HIV
Legionella pneumophila	Legionnaires 'disease
HFRS Hantavirus	Hemorrhagic fever with renal syndrome or Korean hemorrhagic fever
Influenza A virus (H5N1, H7N3, H7N7, and H9N2)	Avian Flu
Filo viruses	Ebola hemorrhagic fever and Marburg hemorrhagic fever
Arena viruses Lassa	Lassa fever
Junín	Argentine hemorrhagic fever

Blood borne Pathogens and Needle stick Prevention [hepatitis B (HBV), hepatitis C (HCV) and human immunodeficiency virus (HIV). Needle sticks and other sharps-related injuries may expose workers to blood borne pathogens], SARS corona virus (Severe Acute Respiratory Syndrome), Legionella pneumophila (Legionnaires 'disease), HFRS Hantavirus (Hemorrhagic fever with renal syndrome or Korean hemorrhagic fever), Ricin (Ricin is a poison that can be made from the waste left over from processing castor beans), Campylobacter jejuni 77.3%, Salmonella 20.9%, Escherichia coli O157:H7 1.4%, and all others less than 0.56% (Food borne Disease), Influenza A virus (Avian Flu) subtypes of avian influenza viruses. These are types H5N1, H7N3, H7N7, and H9N2.

Procedure for Investigation in bio-crimes:
Collection of specimens: The first step is proper collection of evidence at a site where the release of an infectious microbe is suspected. [17].

Reorganization of sample: The next step is recognizing that an attack is occurring and diagnosing the disease. In cases of intentional disease transmission, the identity of the microbe being used in the attack may not be apparent so quickly. Intentional bio attack can be classified as either overt or covert [18-19]. The difference between them is that an overt attack is often recognized immediately; while a covert attack may not become known for some time, if at all. Covert biological attacks are by their nature more difficult to discover than are overt attacks. Indeed, the anthrax letters attack began as a covert attack and became an overt attack with the discovery of the anthrax tainted letters. Regardless, whether an attack is overt or covert, public health officials will likely be the first ones involved.

Analysis of sample: The next step is the analysis of the specimens collected by first responders and by microbiologists subsequently sent to the site. Once collected and preserved, the biological evidence is sent to a laboratory for analysis. Within the laboratory, a core group of analytical tools is available to aid in characterization of the evidentiary material.

Identification: The next major step is the identification of the organism. Microbial forensics is an extension of analysis to microbial agents that are known as bio weapon agents and is primarily intended for identification at strain level for attribution purposes. [20].

Validation of sample: The next step is validating each analytical method by establishing its limitations, its sensitivity and its reliability. The goal is to develop an infrastructure so that microbial forensic evidence will be collected, stored, analyzed, and interpreted in a manner that is scientifically robust and thus legally defensible. Quality Assurance guidelines have been developed [20].

Challenges occur during bio-crime investigations: During bio-crime investigations, number of challenges occurs. Some of them are discussed below:

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- *Collecting specimens at the attack site*
- Recognizing that an attack is occurring and diagnosing the disease
- Analysis of specimens
- Identification
- Validation – Quality assurance and Quality control

Technologies used for molecular identification:
Technologies relied on by microbial forensic scientists need to be properly validated so that the methods used are understood and so that interpretation of results is carried out within the limitations of the assays. The three types of validation are preliminary, developmental, and internal. It uses advanced molecular techniques like microarray analysis and DNA fingerprinting etc. to associate the source of the causative agent with a specific individual or group by measuring variations between related strains. Nucleic amplification and molecular-epidemiological techniques are essential tools in clinical microbiology for identifying pathogens and in outbreak investigations.

To support an investigation, microbial forensic sciences are employed to analyse and characterize forensic evidence with the goal of attribution or crime scene reconstruction. For the analytical part, two very different molecular biology-based assays are described: real time polymerase chain reaction (RT-PCR) [21-31]; these procedures are used to exemplify how molecular biology tools may aid in an investigative process. The first is necessary for rapid response when a threat is imminent or an attack has recently occurred. The latter two apply to implementation of routinely used procedures.

These methodologies can be used to detect and trace back the spread of microorganisms in the context of a crime. Various typing tools have been developed for phylogenetic and phylo-geographic studies. Real-time PCR assays have been developed for the identification of Bacillus anthracis, Brucella spp., Burkholderia mallei and Burkholderia pseudo mallei, Francisella tularensis and Yersinia pestis [32]. Real-time PCR assays are highly specific and sensitive and shorten the time required to establish a diagnosis.

- **Culture and Cytopathic Effect[33]**
- **Electron Microscopy (EM)[34]**
- **Serological Assays** [35-36]
- Enzyme-Linked Immunosorbent Assay (ELISA)
- Neutralization and Hemagglutination Inhibition Assays
- Immunostaining
- **Polymerase Chain Reaction (PCR) [37]**
- Real-Time PCR
- Multiplex PCR
- **Microarray and Virus Chips[38- 40]**
- **Next-Generation Sequencing[41]**
- **Subtractive Cloning[42-43]**

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) [44-47]. 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) [48-50], DNA-sequence based method, for bacterial strain typing [51-52]. 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.

Discussion:
Microbial forensics has been closely associated with the process of attribution, or identifying the perpetrator of a biological attack for purposes of criminal prosecution. Different types of Bacteria, viruses, and fungi threaten the agricultural and food supplies, affect the environment. Pathogens and toxins can be converted to bio weapons and used to commit bioterrorism and bio crime. The use of any of these pathogenic agents as a bio weapon causes serious health concerns to humans. Forensic microbiology is used to determine whether an outbreak may have been deliberate, to trace that outbreak to its source, and to identify those responsible for it.

Forensic microbiological research and education have focused on identifying suspect pathogens to strain level that caused an outbreak. Microorganisms have been used as bio weapons in crimes, although such "bio crimes" are few and can be used to identify the source of the microorganisms used as a weapon and the perpetrator of the crime. The application of forensics microbiology is used to assist in resolving bio crimes, with a focus on research and education in investigations of bioterrorism.

Microbial forensics is one such new discipline combining microbiology and forensic science. It uses advanced molecular techniques like microarray analysis and DNA fingerprinting etc. to associate the source of the causative agent with a specific individual or group by measuring variations between related strains. Microbial forensics employs genetic tools for strain identification. Genomics is increasing the power of microbial forensics by assisting in the design of gene-based diagnostic tests and guiding interpretation. Two different molecular biology-based assays; demonstrate how molecular biology tools may be utilized to aid in the investigative process.

Technologies relied on by microbial forensic scientists need to be properly validated so that the methods used are understood and so that interpretation of results is carried out within the limitations of the assays [53]. Examples of such 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 weaponized the biological agent.

Biological agents include bacteria, viruses, fungi, other microorganisms and their associated toxins. They have the ability to adversely affect human health in a variety of ways, ranging from relatively mild, allergic reactions to serious medical conditions, even death.

Developing systems and methods to detect and track bio crimes will lead to greater safety and security of a nation. An integrated national and international effort needs to be promoted to meet bioterrorism challenges effectively and rapidly.

Conclusion:
This paper describes the role of microbiology in investigative and assessment activities involved in a bioterrorism or bio crimes event. Then an overview is presented of how the field of microbial forensics can assist investigations by analysing and characterizing forensic evidence, with the goal of attribution or crime scene reconstruction.

Microbial forensics is focused on characterization of evidence from a bioterrorism act, bio crime, hoax, or an inadvertent release. If a laboratory scientist suspects bio terror or bio crime, or other legal case, law enforcement agents must be notified and diagnostic samples preserved.

Biological agents are naturally occurring organisms or toxins produced by organisms that can be used against peo-

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ple, animals, or crops to cause diseases. There are hundreds of biological agents, including bacteria, viruses, fungi, and parasites. Exposure to even a small number of organisms can produce severe symptoms or even death. This manuscript provides the ideas about biological agents or bio threats used in biocrimes and provide some facts about different harmful biological agents used in bio terrorism. It also provides abundant evidence that some people have desired to inflict mass casualties on innocent populations through employment of biological agents.

It is evident that even minor bioterrorism incidents have the power to disrupt a large infrastructure of society and lead to a devastating effect on commerce and communication of a country. Better controls are needed to protect legitimate users and to deter criminal dissemination of dangerous microorganisms or their toxic by-products. Better information and/or access to information is required on those individuals who have access to these pathogens so threats can be deterred or effectively traced back to possible sources.

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