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Review

A review on the field of forensic science and different techniques that can be applied to identify the pathogen and perpetrator of a bio crime

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Abstract: Microbial forensics is a developing field that involves collecting evidence from the scene of a bio crime to identify the pathogen used, as well as building a case for criminal prosecution against the perpetrator. The field of microbial forensics and forensic science in general is continuously advancing to utilize the best equipped technology to collect microorganisms and potential clues from a crime scene as well as tools to test these samples and identify harmful microbes as well as the perpetrator. The first crucial step in a bio attack is collecting the microorganism with a variety of methods being available. Identifying a microorganism after collection is also crucial and is often done by genome sequencing and DNA typing. Decontamination is necessary to keep the public safe, and methods such as foam, chlorine dioxide gas, and methyl bromide fumigation have shown promising results in disinfecting a contaminated area. The identification of a perpetrator in a bio crime is important for prosecution and studies using techniques such as DNA typing, short tandem repeat (STR) analysis, and polymerase chain reaction (PCR), show efficacy in identifying the origin of an attack. Bio crimes have the potential to be dangerous to the public, and for that reason, various efforts have been made to advance the field of microbial forensics. There are many promising studies, moreover, that show efficacy in combating a biological attack. To protect the public, implementing a system of preventative measures is crucial.

1. Introduction

While many people are familiar with the field of forensic science through television shows and the media, the specific field of Microbial forensics is a newly emerging discipline that many may not be as familiar with. Microbial forensics is an area of forensics in which physical evidence from the scene of a bio crime or bioterrorist attack is analyzed for the purpose of criminal prosecution (1). With many biotechnological and overall scientific advancements in the field of forensics, microbiology has become a key aspect in the investigation of legal matters (2).

A bio crime is a crime in which the weapon is a microorganism that poses danger to the public (2). Furthermore, this microorganism can present as a pathogen, virus, or toxin, all of which are applied for the expressed purpose of intentionally directing harm to a person or even a large group of people (2). DNA typing, a laboratory method that detects variation in DNA, is used in the field of forensics to identify a perpetrator of a crime, usually a sex crime or murder (Bittner 2004). DNA typing is done through various techniques; however, the key is identifying variation in the DNA sequence (3). This method of identifying a person responsible for a crime is much more challenging when the crime is a bio attack (2). For this reason, the field of microbial genomics is still developing and not as well established as other areas of the forensic science field (4). Bio crimes are few and far between in comparison to other crimes but using forensics to not only identify the pathogen used but also the perpetrator is important (2).

With microbial forensics being a newly emerging field, there are many challenges yet to be resolved. Insufficient understanding of microbial diversity is a major challenge

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in determining from where bio attacks, bioterrorism, and bio crimes are initiated (5). A better understanding of the pathogens or toxins present in a bio attack is necessary to determine perpetrators in bio attacks (5). One study suggested the development of other than genomic approaches such as multitarget analysis of culture media which may be useful for the purpose of deeper forensic characterization (5). Furthermore, political and governmental constraints limit shared global standards and databases for Microbial forensics and genomics which lessens the confidence of microbial forensics analysis (5).

In this review we will examine how microbial forensics can be used to: 1) identify a pathogen or toxin in a bio attack, 2) decontaminate the crime scene, and 3) identify the perpetrator. One study found that qPCR is useful in enhancing the detection of bio contaminants on a surface and identifying the best sampling method (Buttner et al. 2004) while another study found that using a biological sampling kit (BiSKit) to test large surface areas is more efficient and yields more accurate results than other methods (6). A study that looked at decontamination strategies found methyl bromide fumigation is able to successfully decontaminate *B. anthracis* spores from various surfaces in a building with no damage to the edifice or its contents (7). Researchers also find that short tandem repeat DNA typing can be used to identify a perpetrator of a crime and this paper will discuss how this can be applied to bio crimes specifically (8). This review will explore the landscape of a bio crime and how to address different issues to keep the public safe.

Some examples of well-known bio attacks are the typhoid fever of 1984 and the anthrax attacks following the attack on the world trade center on September 11th, 2001 (9, 10). The typhoid fever, caused by Salmonella typhi, led to hundreds of people who were infected with the bacteria in 1984 after intentional contamination of salad bars in Oregon (9). These salad bars were contaminated with the intentions of impacting local city elections but thankfully no one died as a result of the attack (9). The anthrax attack is another example of a well-known bio attack (10). In this case, letters contaminated with anthrax were mailed to a variety of offices such as news media offices and the offices of public officials (10). As a result, 17 people were infected, and 5 people died (10). Groups and individuals can use biological weapons as a way to inflict terror on people as well as have social and political effects on society (9).

Collection of the microorganisms at the scene of a bio crime

The collection and identification of microorganisms following a potential bio crime is not only a crucial first step in being able to piece together information about a crime, but it is also important in understanding the potential threat to people immediately affected as well as the general public. One study found that qPCR has the ability to enhance the detection of bio contaminants on various surfaces and is helpful in comparing and identifying the most useful of different sampling (11). There are various methods to detect microorganisms on surfaces yet not all are as well equipped to accurately identify and enumerate microorganisms (12). While swabbing is a highly used method its success is largely dependent on the skills of the person collecting the samples and is therefore prone to human error (13). The use of contact plates, which is the direct contact of an agar plate with a contaminated surface is slightly more useful, but problems often arise if the surface in question is rough, wet, or has any other quality that can potentially adversely affect growth on the plate (14). In addition, these sampling methods are really only successful for sampling small areas, which can be tedious and time consuming because sampling of a small area requires a greater number of samples to be collected and this can delay the reporting of results (6). In addition to this, because a small surface area is being sampled, this means that the total number of microbes recovered will be lower than if a larger surface area was being sampled (6). Both of these challenges make it more difficult

to identify a potential pathogen in the case of sampling methods that rely on a small surface area (6).

A proposed solution to this issue is the biological sampling kit (BiSKit). The kit is designed to sample a surface by wet and dry methods using either a cotton-based swab or a foam-based swab and these resulting samples are analyzed by culturing, quantitative PCR (qPCR), and immunological assays (6). The BiSKit is used to sample a large surface area ($1m^2$) and is compatible with various forensic technologies (6). Buttner et al 2004 found that the BiSKit was not only more efficient than the swabbing method but also resulted in a 10-fold increase in microbial concentrations obtained indicating that this is a successful sampling method that improves sample size and the number of microbes captured (6).

Identification of the microorganisms

While genome sequencing and DNA typing have long since been used to identify the perpetrator(s) of a crime, these technologies can also be used to identify microorganisms used as weapons in bio crimes. Genome sequencing, DNA typing, and next generation sequencing (NGS) allow scientists to type bacterial strains in a way that does not involve phenotypic assays (15).

Comparative genome sequencing was used following the bioterrorist anthrax attack in 2001 (16). Genome sequencing was able to trace anthrax samples back to distinct bacterial families, which is significant in identifying a pathogen, and at the time was a powerful new tool in the investigations of highly variable genomic regions and revealed that all of the infected letters delivered to the various locations came from the same source. This was a huge breakthrough in the case (16). However, while genome sequencing and comparisons can be used to identify bacteria, these techniques are more costly and time consuming than bacterial DNA typing (15).

There are a variety of DNA typing techniques and the one technique that is efficient and effective and can be useful in the identification of pathogens in a bio crime is automated repetitive-sequence-based PCR (rep-PCR). This technique uses primers that target non-coding repetitive sequences in the genome (11). It is easy to perform and an effective method for bacterial strain typing. (11).

A more recent development in the area of microbiology that can also be used in the identification of microbes is next generation sequencing. Whole genome next generation sequencing (WG-NGS) is a technique that has revolutionized biology with improvements in sample preparation and bioinformatics pipelines, and the decrease in cost (17). WG-NGS allows for the deep sequencing of nucleic acids, the identification of anomalies, patterns and correlations within datasets, as well as the analyses of a variety of other characteristics (17). This technique is able to provide more detailed taxonomic information than diagnostic PCR (17).

Decontamination of the area

Bio crimes can have harmful consequences and be propagated rapidly, and therefore, measures to mitigate crimes must be fast acting and efficient. When a bio crime is committed, it is crucial to decontaminate the area affected by the microorganism(s) (18). The microorganisms can contaminate a small surface area, an entire room, or even multiple buildings (7). The first step in decontamination is understanding what microorganism infected the area (7). A study conducted by Buttner et al. 2004 used an experimental room with office furniture, containing materials like wood, metal, and tile. The rooms were contaminated with endospores of *Bacillus atrophaeus*, and treated with two different products, foam decontaminant and chlorine dioxide gas, that when mixed together, are composed of cationic detergents, fatty alcohols, stabilized hydrogen

peroxide, water, and inert ingredients (19). When quantitative PCR was used to sample the room before and after treatments, *B. atrophaeus was* still present after treatment, however, the levels of culturable *B. atrophaeus* were reduced immensely (19). Buttner et al 2004 concluded the foam treatment is effective in that it left no culturable strains of *B. atrophaeus* and reduced the contaminant by 5 orders of magnitude. For the Chloride dioxide treatment, the study concluded it too was effective and reduced the contaminant by 4-5 orders of magnitude (19). While the not 100 percent efficient, the two treatment modalities significantly reduce the contaminant (19)

The study conducted by Buttner et al. 2004 illustrates the effectiveness of two methods that can be used in decontaminating an indoor setting with furniture; however, with any method there are always downsides. Buttner et al. 2004 discuss the disadvantage to chloride gas decontamination as the need for specific gases and equipment that can only be used by trained personnel, therefore accessibility may be an issue depending on where the bio attack is committed.

Another study performed by Calfee et al. 2015, showed the efficacy of methyl bromide (MB) fumigation. MB fumigation was conducted to decontaminate *Bacillus anthracis.* The building contaminated with the pathogen was sealed under two tarps and fumigated at ≥225 g m^{-3} mean concentration for 48 h at 28°C (7). Calfee et al 2015 found that the MB fumigation allowed for the complete removal of *B. anthracis* without damage to other objects in the room including electronics and furniture. Furthermore, MB fumigation is fast, economical, and effective (7). The studies discussed have proposed orderly and beneficial matters that fumigate an infected bio crime area.

Identifying the perpetrator

Lastly, identifying the perpetrator is an important aspect of the microbial forensic investigations. Scientists can also use microbial forensics to identify the offender(s) of the crime. When intentional bio crimes occur, they are typically categorized as: 1) overt, meaning the attack is recognized immediately, or 2) covert, meaning the attack is unknown (21). DNA typing is commonly used in the process of identifying an individual as a perpetrator (22) and is an umbrella term that represents the different methods used to study DNA variation (23).

More than 99.5% of the human genome is the same throughout the population, it is the other 0.5% of DNA that shows variation between individuals, which allows for identification (22). One method of DNA typing that is often used with humans is short tandem repeat (STR) analysis. STRs are 2-6 bp DNA sequences that are repeated continuously, throughout the genome (24). STR DNA typing detects differences in the repeated lengths of DNA sequences (24). To analyze the DNA, STRs are amplified using PCR (24). The STR amplification products are then sized using capillary electrophoresis or some other method (24). The size of the STR repeats is key to identifying the perpetrator(s) because there is a very remote chance that two individuals share the same STR lengths (25). However, with the continual advances in technology, a more standardized method has been developed using fluorescent detection of PCR fragments (24). This is done through capillary electrophoresis of PCR fragments (24). Huber et al. 2008 developed a multiplexed PCR assay, allowing for simultaneous analyses of different STR loci. Multiplexing is important because the additional information increases the statistical power and helps scientists particularly in cases when the DNA is degraded (24).

The goal of finding the origin of a bioagent helps in identifying individuals involved in crime (21). Tracing or Surveillance is also a key factor in linking a perpetrator to a bio crime (21). Because each bio crime is unique, different approaches are undertaken and are dependent largely on the collection of evidence (21). For example, different approaches are specific to hairs, fibers, fingerprints, or in the case of a covert attack,

evidence may be limited to using medical histories, diagnoses, and isolates taken from victims (21). With the evidence at hand, genetic markers are used to match DNA samples, allowing for a foundation in finding a perpetrator (21). Then, further steps are taken such as the ones indicated previously in this section (PCR, STR), to independently validate results. After analyzing the evidence, DNA databases that are standardized through the FBI are used to compare and match the sample DNA (26). This system is known as CODIS--combined DNA index System (24).

Discussion

Infectious outbreaks can be a national or even global threat and lead to serious illness, death, and public anxiety (27). While these outbreaks can be due to natural influences there are others that can be intentional with the purpose of harming specific people or even the general public (27). These crimes are referred to as bio crimes or bioterrorism and while infrequent they can potentially be highly dangerous to the public (27). The purpose of this review was to outline ways to identify pathogens and the perpetrator as well as effective ways to safely clean up the crime scene and eliminate the threat. There are a variety of methods used to safely collect the pathogen and later identify it, some of which are better than others. There are also different methods that can be used to decontaminate the area for the purpose of protecting the public. Identifying the perpetrator for the purpose of criminal prosecution is also important and a variety of different methods for all of these different aspects of bio crimes were outlined in this review.

The first step in being able to treat people who may have been infected by a bio crime as well as keeping the rest of the public safe is identifying the pathogen used in the crime. Before the pathogen is identified, the crime scene is sampled. A variety of methods for sampling surfaces were outlined in this review. Some methods were noted as being more effective and efficient than others. The swabbing method which is commonly used is prone to human error (13) whereas contact plates are only useful if the surface is flat and dry (14). Another issue is that regardless of the specific method used, most have only typically been used to sample small areas (6). The BiSKit can be used to solve this problem (6). This sampling method can be used to collect samples from large surface areas making it more efficient. It is also compatible with a variety of technologies (6).

After collecting from a crime scene, samples are tested to identify the pathogen. Studies on genome sequencing and DNA typing were reviewed. Comparative genome sequencing was a method widely used in the 2001 anthrax attacks and was significant as this was how the strain of bacteria was identified (16). While this is significant, genome sequencing is costly and time consuming in comparison to DNA typing. Because of this DNA typing was looked at next (15). There are different DNA typing techniques, one being rep-PCR which is easy to use and efficient in identifying specific pathogens (3). Another technique WG-NGS is noteworthy as it allows for more detailed taxonomic information than diagnostic PCRs (17).

After identifying the pathogen in the bio crime, decontamination of the infected areas is key to preventing further outbreak. In a study that aimed to decontaminate the pathogen *B. atrophaeus,* foam treatment and a chlorine dioxide gas treatment were used, and this combined agent showed efficacy in the removal of this specific pathogen *(*19). Another study used MB fumigation to combat the pathogen *Bacillus anthracis.* With high concentration of MB in a sealed and tarped building, the experiment indicated efficacy of the treatment to disinfect the area (7).

Identifying the perpetrator of a bio crime is another step in the microbial forensic investigation. DNA typing is commonly used in identifying a perpetrator and can be accomplished using various methods. One of the most frequently used techniques is the amplification of STRs, which looks at the DNA sequences of 2-6 bp that are repeated in

long stretches across the genome (24). STR amplification is a powerful DNA typing method that makes use of fluorescent detection of PCR fragments via capillary electrophoresis (24).

Forensic science is still an emerging field with room for significant advancement. Many of the technologies used in the field and mentioned in this review are outdated. Distinguishing between intentional versus unintentional bio crimes is a challenge. In the cases of a covert attack, initial sampling evidence may be limited to medical histories, diagnoses, and isolates taken from victims, making it difficult to identify the perpetrator(s) (21). These presented challenges indicate that further research is necessarily in order to mitigate bio crimes.

Tools to better predict or prevent bio attacks are urgently needed. For a biological attack to occur, there must be a vulnerable target, a person/group with the ability to attack, and the intent by the offender(s) to carry out the attack (28). Unfortunately, as seen repetitively throughout various studies, there is no standardized protocol for bio attacks. This is mainly due to the issue that every possible bio attack presents itself in a different way each time; and therefore, methods to combat the specific bio attack vary from one another. However, prevention needs to become a key role in stopping further bio crimes. Therefore, more research on successful and effective preventative measures of a bio crime is necessary to ensure public safety from harmful bio agents.

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