Chapter 13
The Role of Bioforensics in Medical Bio-Reconnaissance

Lothar Zöller and Gelimer H. Genzel

Abstract Since the 1990s, a broad spectrum of regional conflicts and crises have evolved that have been accompanied by a growing threat of international terrorism. How vulnerable our modern societies would be towards a covert biological attack became evident in the 2001 anthrax letters attack in the United States. Biothreats are currently associated with asymmetric warfare scenarios and non-state actors rather than with state-driven biowarfare facilities. Against this backdrop, NATO has to consider biological warfare and bioterrorism as a serious threat to its forces. In bioterroristic scenarios the deliberate release of a biological agent will most probably remain undetected until a cluster of cases will suggest an unusual outbreak of disease. In military settings it is primarily the responsibility of the Medical Services to recognize the outbreak and to launch an appropriate outbreak investigation. Major goals of a medical bio-reconnaissance mission are to rapidly identify the causative agent of the outbreak and to differentiate between natural and deliberate outbreaks. In contrast to the investigation of overt natural outbreaks, forensic aspects have to be considered and appropriate procedures have to be implemented quite from the beginning when unusual outbreaks are to be investigated. If a biothreat agent is detected, it may be necessary to enter further genetic analysis in order to differentiate between natural and intentional outbreaks and to trace back the origin of the agent. Microbial forensics is mainly concerned with taking molecular fingerprints of biothreat agents by means of molecular typing techniques enabling the investigator to identify and trace back a particular strain by comparing it with the fingerprints stored in a typing database. The bioforensic approach may well be capable of elucidating the source of an outbreak as has been evidenced in the Amerithrax case in 2001. In order to detect molecular differences of microbial strains, a number of sophisticated typing techniques are currently employed, the most recent of which is whole genome sequencing, which has even entered the field laboratories by means of portable next generation sequencing devices like the MinION™.

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13.1 Approach to the Term Bioforensics

The term "bioforensics" or "microbial forensics" came into common use during the investigation of the anthrax letter attacks in the United States in 2001 although it had been mentioned already years before by FBI forensic experts. The investigational approach, which is also known as the "Amerithrax" investigation, was unique at that time in involving criminologists, namely the FBI, as well as microbiologists and genome sequencing experts [1, 2]. Their common goal was to trace the anthrax bacilli used in the attacks back to their source and to link it to the perpetrator, which overall took 9 years of investigation. This kind of trace-back analysis to solve a biocrime or a bioterroristic act gave name to a new scientific discipline called microbial forensics [3]. The approach is very similar to the bundle of methods that are nowadays often applied to trace the sources of natural infectious disease outbreaks, which is also known under the term "molecular epidemiology".

Generally spoken, bioforensics or forensic biology applies the biological science to the investigation of legal matters. The goal of bioforensics is to answer the question who committed the offense. In that logic microbial forensics is a subdiscipline of bioforensics dealing with microbes and their characteristics as evidence in a criminal investigation. It has turned out to be a helpful instrument in cases where microbes have been used as a weapon, but may also be useful in cases where microbes provide a characteristic signature on evidences, thus helping to trace their origin. But, during the anthrax letter attacks in 2001 the delay in the diagnosis of the first cases and the long duration of the molecular trace-back analysis, which finally led to the identification of the perpetrator, also revealed the gaps in responding to unusual biological events.

Even though the term bioforensics obviously describes a wide and complex scientific field, the official NATO Glossary is using the term "CBRN forensics" in a more general way to designate "the scientific methods and techniques used to analyze materials and data in support of a chemical, biological, radiological and nuclear incident or threat investigation" [4]. In a military setting bioforensics as a subdiscipline of CBRN forensics designates a complex of procedures and scientific methods that allow the discrimination between a biological attack and a natural outbreak and the attribution of a biological attack to the perpetrator. Bioforensics is part of bioreconnaissance, which in a wider sense designates all measures that aim to detect, recognize, trace back and attribute the deliberate release of a biothreat agent. Biothreats are currently associated with asymmetric warfare scenarios and non-state actors (bioterrorism) rather than with state-driven biowarfare facilities. In bioterroristic scenarios a covert release of the biological agent must be expected, which will most probably remain undetected until a cluster of cases will suggest an unusual outbreak of disease in the target population. This is why a biological attack will most probably manifest in a way that in the first instance will involve the medical facilities and, in a military setting, the Medical Services of the Armed Forces. Samples recovered from the casualties will not only be needed for a rapid and proper diagnosis but will also constitute pieces of evidence in an investigation that might result in serious legal, political or even military consequences. The Armed Forces Medical Services must therefore become aware of this new role they are playing in case of a biological attack and implement appropriate forensic procedures. Over the past 10 years the Medical Service of the German Armed Forces has built up a concept to implement bioforensic procedures in its bio-reconnaissance facilities, which is outlined in the following.

13.2 Principles of Bio-Reconnaissance

13.2.1 Natural vs. Intentional Outbreaks

It is highly unlikely that the deliberate release of a biological agent in the framework of a biological attack would be detected before the agent would reach the target population or infrastructure. Due to the variety of potential biological agents and associated routes of delivery as well as the need to distinguish them from non-human pathogens, current biomonitoring systems are far from providing reliable real-time detection that would allow to implement protective measures or even to warn the target population in due time [5]. Only in scenarios where a biothreat is recognized prior to the use of bioweapons, i.e. where an enemy threatens to use bioweapons or is known to possess them, aerosol detectors may be of value in providing indications of the release of airborne biological agents. This may allow to implement post-exposure prophylactic measures or early treatment during the incubation period (detect-to-treat capability).

An outbreak is basically defined as the occurrence of two or more cases of a disease or deaths that are closely related in epidemiological terms. Minor local outbreaks of an infectious disease often mark the initial phase of an epidemic. An outbreak can be characterized as "unusual" when its ecological, epidemiological, infectiological and microbiological characteristics are non-standard. "Standard" would be the typical occurrence of an infectious disease with the expected seasonal, geographic and demographic distribution patterns and the known clinical picture. However, the deliberate release of a biological agent must be suspected if the epidemiological, microbiological or clinical features of the disease are unknown, new, unexplainable or unexpected, for example if the pathogen is not known to be endemic in the area where the outbreak occurs. Atypical courses of disease that differ from commonly seen natural infection, e.g. inhalational anthrax or primary pneumonic plague, should also raise suspicion as should unusually high rates of manifestation and lethality. A non-natural cause of a disease may also be suspected in case of an unusually short incubation period or unknown or uncommon antibiotic resistance properties of an endemic pathogen. Intelligence or police evidence of a biological threat may provide additional clues. The intentional release of a biological agent would be even harder to reveal if a previously unknown or genetically engineered agent or a regional endemic pathogen were disseminated. In the latter case, the outbreak could even simulate a "natural" epidemic. Therefore, it is a basic need for the military Public Health facilities to continuously monitor the epidemiological situation of infectious diseases in the theatre of operation and to report anomalies strictly according to the requirements specified by each nation [6].

Biological warfare agents usually need hours to days to take effect after hitting the target population. Incubation period and clinical manifestations depend on the type, virulence, quantity and delivery of the agent as well as on the susceptibility of the exposed population (e.g. vaccination status, underlying diseases). The appearance of an outbreak with an unusual disease manifestation or an unusual epidemiologic pattern is, therefore, the most apparent clue to a covert biological attack. As soon as an outbreak has been recognized an outbreak investigation must be put in place, the first major goal of which is to identify the causative agent because in a proverbial sense "you cannot win the war if you don't know the enemy". The second major goal is to identify its potential source, e.g. food, water, animals or others. This is, by the way, equally important for natural as well for deliberate outbreaks.

If the outbreak is characterized as unusual, it will always be necessary to implement efforts to discriminate between a natural outbreak and an intentional event. A scoring system has been proposed that may give a first assessment and trigger further investigations [7, 8]. It will also be important in such a case to supplement the investigation with forensic procedures to preserve evidence.

13.2.2 Recognizing an Outbreak

After a covert biological attack an uncommon disease outbreak and/or sudden deaths will primarily alert rescue services, emergency physicians, resident physicians, internists, specialists in tropical and laboratory medicine, microbiologists and pathologists as well as doctors and nurses of outpatient clinics, emergency rooms and hospitals. Pharmacists may notice a sudden increase in antibiotic prescriptions. Epidemics in animal populations that may also be targets of biological attacks will first be recorded by animal owners and resident veterinarians and will be reported to the responsible animal health offices. Early detection of an outbreak largely depends on the awareness of physicians initially contacted by the patients and the public health facilities behind them whose responsibility is to recognize an outbreak and to put in place adequate epidemiological methods for outbreak investigation. In a military setting, physicians should, therefore, be routinely trained to recognize infectious disease outbreaks and to call in biodefence specialists as soon as the outbreak reveals "unusual" characteristics. In the Medical Service of the German Armed Forces this is a routine part of the postgraduate training of all physicians. A case definition must be developed early during the outbreak investigation in order to help first responders and physicians to recognize further cases and to provide a differential diagnosis. Because different biological agents may have been used

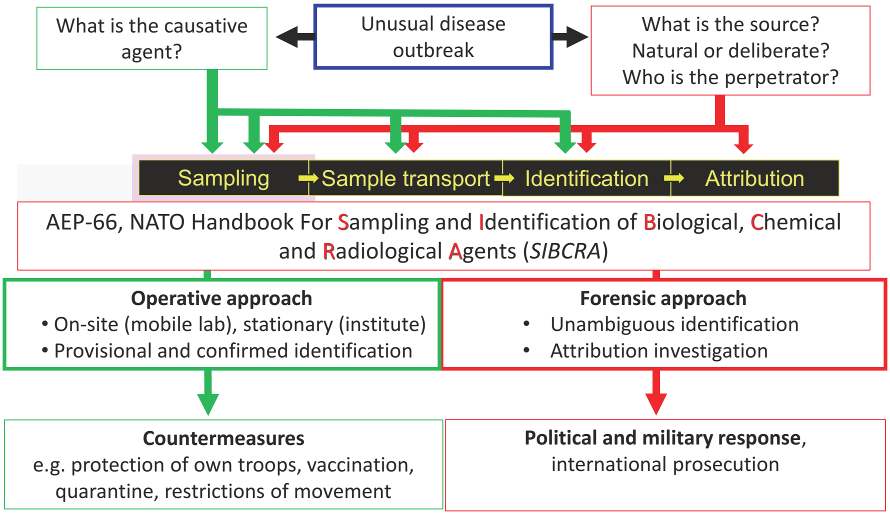

Fig. 13.1 Principles of bio-reconnaissance in the Medical Service of the German Armed Forces: operative and forensic approaches

simultaneously or at different times and in different ways (e.g. airborne, alimentary), the triage process must also consider different possible syndromes if applicable.

13.2.3 Outbreak Investigation in a Military Setting

13.2.3.1 Responsibility of the Medical Service

The bio-reconnaissance facilities of the Medical Service of the German Armed Forces comprise medical bio-reconnaissance teams who are trained to enter a scenario under adequate personal protective equipment, to assess it, and to recover the proper specimens. Standard operating procedures for the qualified recovery of samples from humans, animals, food and water and for appropriate sample transport under strict observance of forensic requirements have been established. The German medical bio-reconnaissance facilities also include methods for epidemiological investigation, e.g. to conduct a case control study in collaboration with military public health experts, and also methods for the unambiguous identification of the causative agent and for molecular trace-back analysis. The Medical Service of the German Armed Forces is, therefore, prepared to make decisive contributions to achieving the major goals mentioned in the previous section of this chapter.

In what is called an operative approach (Fig. 13.1), identification of the causative agent is primarily the responsibility of a field deployable laboratory, which is a modular system of devices, assays, protective equipment and trained staff that allows to perform diagnostic testing from specimens on-site at the level of provisional and in part confirmed identification. All the laboratory and personal protective equipment is packed in handy carrying cases and can be deployed by military or civilian aircraft within 48 h to any place where the action is needed [9]. The field lab is supported by the stationary reach back facilities of the Bundeswehr Institute of Microbiology (BwIM), which extends the level of confidence in agent identification to the levels of confirmed and unambiguous identification. Proper identification of the causative agent will always be a prerequisite for the implementation of adequate countermeasures such as vaccination, chemoprophylaxis, quarantine or restriction of movement. It is very time-sensitive, because the "window of opportunity", during which the outcome of the outbreak can be significantly influenced, is rather short.

In addition to this, in case of an unusual outbreak that might have been caused by the intentional release of a biothreat agent, a "forensic approach" (Fig. 13.1) must be implemented quite from the beginning and throughout all stages of the diagnostic process from sampling to identification.

13.2.3.2 Sampling

Sampling is a pivotal step in the first phase of an outbreak investigation. There might be only one chance to recover adequate samples from humans, animals (living or dead) or from the environment, which will be the basis for all consecutive laboratory examinations and the conclusions drawn from the results. This is especially important if the outbreak might have been caused by a biological attack. Forensic procedures must, therefore, be implemented quite from the beginning in order to preserve evidence and to safeguard the chain of custody. Insufficient procedures and flaws during the sampling process due to a lack of expertise cannot be compensated at a later stage of the investigation. Thorough photographic documentation of the sampling situation and the use of pre-controlled lots of sampling devices are mandatory. Samples should always be taken in duplicate. In order to transport the samples to a laboratory, sample vessels must be sealed by means of security labels wearing individual numbers. These labels display immediate evidence of attempted removal or manipulation. Furthermore, each sample must be wrapped in a special decontaminable transportation pouch. Thorough documentation of the sampling sites and samples must be done by setting up a record on tear-proof and autoclave-resistant paper. A major task of the sampling team is to safely and securely transport the samples to the lab by using appropriate transport vessels and boxes fulfilling the requirements of the international dangerous goods regulations. It is important to safeguard the chain of custody during the whole bio-reconnaissance process, which means that the transport chain of the samples must be fully supervised and documented from the sampling sites to the lab. To give an example of this, the deliverer of the specimens must be photographed with his identity card in hand (Fig. 13.2). Throughout the sampling, transport and subsequent laboratory analysis processes, the legal integrity of samples and data must be

Fig. 13.2 Safeguarding the chain of custody: photo-documentation of sample delivery to the laboratory

guaranteed. Therefore, it is necessary to have an accurate written record to track the possession, handling, and location of samples and data from collection through reporting.

13.2.3.3 Mobile Laboratory Investigation

The German rapidly deployable laboratory facility is designed and equipped especially for the reconnaissance of intentional outbreaks. It is based on a modular concept of devices and assays that allow sampling and on-site diagnosis in various situations. The lab can be operated in multiple environments, even in a garage, but is also equipped with an inflatable lab environment to allow autonomous operation. Most of the diagnostic procedures are realtime-PCR-based and currently adapted to the SmartCycler™ (Cepheid Inc.) platform, but the lab also provides methods for light microscopy and antigen detection (Enzyme immunoassays, immunofluorescence assays, handheld testkits). Cultivation techniques are not provided at the level of the mobile lab. During the handling of potentially hazardous and forensically important samples in a mobile laboratory, appropriate biosafety precautions like the use of a low-pressure vented glovebox must be observed, not only to protect the laboratory staff from infection, but also to avoid any contamination that could later on lead to misinterpretation of the results. This is especially true for the sensitive step of DNA- or RNA-extraction. Retention samples should be saved from crucial steps in order to preserve the option to control doubtful results. Therefore, only highly trained and experienced laboratory and scientific personnel must operate such a diagnostic field laboratory.

13.2.3.4 Identification of the Agent

Arriving at the identification step, it is important to implement the highest possible level of confidence, which can be achieved by the combination of independent methods and independent diagnostic targets. The NATO SIBCRA Handbook [10] differentiates between the levels of provisional identification, which basically means the use of one method aiming at antigen detection or nucleic acid detection or cultivation of the agent. Confirmed identification requires at least two methods and unambiguous identification requires all three procedures and, if possible, an additional animal experiment to prove the pathogenicity of the agent. The latter level is, of course, reserved for stationary labs.

To ensure the quality of the diagnostic procedures and algorithms but also the evidential value of the results, accreditation of the stationary laboratory according to one of the pertinent standards, e.g. the European standard 15189 for medical labs, is mandatory as is the implementation of a quality management system in the mobile laboratory facilities.

13.2.3.5 Trace-Back Analysis and Attribution Investigation

Bioforensic attribution investigation and trace-back analysis of the source of the agent relies predominantly on the genetic analysis of the outbreak strain. In principle, it's taking a genetic fingerprint from the outbreak strain and comparing it with stored fingerprints of other strains of the same species by means of bioinformatic databases, into which the data of the individual strains have been integrated. The fingerprints can be linked to other available information on the individual strains like geographic regions where they typically occur, the strain history or known genetic characteristics of the respective species, e.g. clonality or mutation rates.

Various molecular typing methods can be used for this purpose [11], the most recent of which and the one with the highest discriminative power is whole-genome sequencing, which has become affordable and is a routine method now for outbreak analysis [12] in specialized lab units as BwIM's Microbial Genomics and Bioinformatics Group.

Bioinformatics is a discipline that contributes crucial and indispensable techniques to microbial forensics, e.g. algorithms for large-scale genome alignment and comparison or for the determination of gene composition, protein structure and the functional organization of genomes. It also provides methods to analyze the mechanisms and markers of pathogenicity and antibiotic resistance. The principal goal of bioinformatics in microbial forensics and diagnostics is to identify and characterize microorganisms at various levels of resolution (genus, species, clone, strain, substrain).

Microbial forensics does not only have the capacity to elucidate the origin of an outbreak strain, but also to reveal possible genetic manipulations that may be intended to enhance virulence or to introduce unexpected antibiotic resistance properties [13].

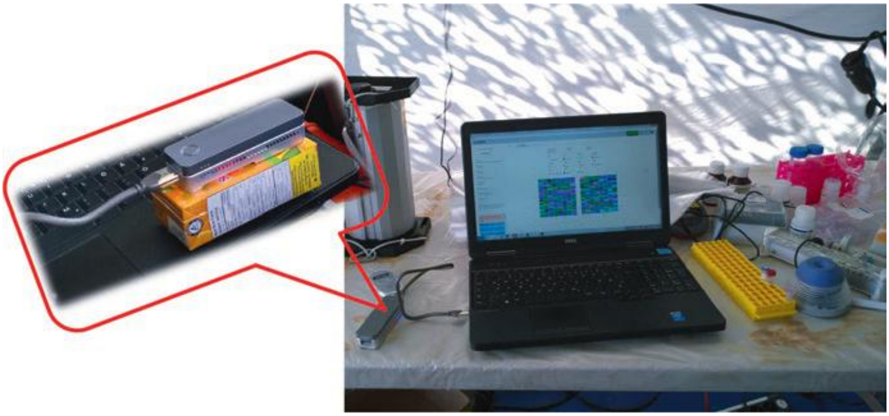

Fig. 13.3 The MinION™ sequencing device in the field laboratory during a NATO exercise

The latest development in microbial forensics is the use of portable third-generation sequencing devices like the MinION™ in the field [14], which allows sequencing independently from stationary laboratories and in close proximity to the epicenter of the outbreak (Fig. 13.3).

The value of such a trace-back analysis can be exemplified by an outbreak of anthrax in drug addicts caused by spore-contaminated heroine that had started in Scotland in 2010 and, after hitting several other countries, erupted in Bavaria in 2012. The method employed to investigate the outbreak strains was SNP analysis, which allows the attribution of strains to branches, clusters or groups by the analysis of single marker nucleotide positions in the genome that have changed during the course of evolution and have become characteristic for each clade. The outbreak strain could be attributed to a defined subcluster of the so-called A-strains. The closest relatives of the outbreak strain could be localized in Eastern Turkey close to the Afghan border, which is in concordance with the known route of heroine from its production site in Afghanistan via Turkey to Europe. Most probably the site where the contamination took place is somewhere in Eastern Turkey or in Afghanistan itself [15].

For the sake of completeness it should be mentioned here that besides the genetic signatures there are also chemical and physical signatures that may be relevant in microbial forensics [16]. For example, it is a proven fact that the chemical makeup of microorganisms (e.g. spores) reflects the environment (e.g. the medium) in which they have grown. Differences in the composition of media may, therefore, influence the protein profiles of the spores of Bacillus strains cultivated in them. Chemical substances added to media or spore preparations to ease aerosolubility may also provide forensic signatures. Methods to reveal such signatures include mass spectrometry and electron microscopy to name but a few. We are not going deeper into these aspects here because it is beyond the scope of medical bio-reconnaissance, which deals with patient specimens rather than with environmental samples. Microbial isolates cultivated from patients or animals will probably have lost the chemical and elemental signatures they may have originally had in the bioweapon. One of the major future challenges in the field of microbial forensics will be the implementation of a quality management system in order to ensure the legal usability of the data.

13.3 Conclusion

After the fall of the Berlin Wall in 1989 and entry into the "War on Terror" in 2001, the biothreat situation has fundamentally changed. A large-scale attack with airplanes spraying clouds of biological agents or throwing toxin-filled bombshells, the classical Cold War scenario, is no longer a major threat in a world where state-funded bioweapon programmes have largely been given up, although some states may still pursue hidden bioweapon programmes. Current threat analyses by intelligence services and security organisations instead point to the use of bacteria, viruses or toxins by personally (*biocrime*) or politico-religiously (*bioterrorism*) motivated groups or individuals. Modern biological defence, therefore, focuses on the development of concepts and strategies on how to minimize the damage caused by such scenarios to the population and/or to military forces. Early recognition of an attack is crucial as is the implementation of forensic procedures during the whole process of bio-reconnaissance. To prove a biological attack in a way that the investigative evidence is usable by the International Criminal Court or would justify a political or even a military reaction is, however, challenging. And, surprisingly, the paradigms have changed, as bio-reconnaissance is no longer a matter of the CBRN defence forces alone but requires sophisticated facilities of the Medical Services. Hence, this chapter outlines the rationale for the Medical Services of our NATO Armed Forces to enhance efforts in the field of bioforensics.

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