Bacteria as potential tools in bioterrorism, with an emphasis on bacterial toxins

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ABSTRACT

The threat of bioterrorism remains a reality worldwide and, although of low probability, an attack would be a high-consequence event. Microbes are available to individuals with appropriate contacts and even many low-grade bacterial pathogens can severely affect health. Toxins provide bacteria with a system of defence that is often detrimental to humans and their versatility makes them potential tools of bioterrorism. It should be remembered that the aim of terrorism is not always to kill but rather to strike fear into peoples lives. Therefore, agents such as botulinum and cholera toxin could be used, which may not cause significant mortality but would cause widespread panic and potentially high morbidity. Importantly, no state can ever be fully prepared for a response and it is probable that no state ever could be. It is for this reason that biological agents are so attractive as weapons.

KEY WORDS: Anthrax. Bacteria. Botulism. Toxins. Violence.

Introduction

Agents of bioterrorism are not limited to the high-profile list seen in the newspapers, magazines and on the television. A number of common pathogens can also be used as instruments of terror, while in the future others may be manipulated by modern molecular biology techniques to make them spread more easily, be more virulent and more resistant to antibiotics.

New infectious agents continue to emerge, as seen recently with the severe acute respiratory syndrome (SARS)[1] and avian flu[2,3] outbreaks, and the deliberate release of agents that can cause significant veterinary or agricultural damage should not be ignored. Not only do such releases have potentially devastating effects on the food supply but the indirect costs can be very high. This review illustrates examples of bacterial toxins as potential agents of bioterrorism, while some historical aspects and the impact of bioterrorism are also described.

Brief history of bioterrorism

Agents of biological warfare can be defined as "living organisms, whatever their nature, or infected material derived from them, which are used for hostile purposes and intended to cause disease in man, animals or plants, and which depend for their efforts on their ability to multiply in the person, animal or plant attacked".[4]

Despite the relatively limited number of publications on bioterrorism prior to 1998, bioterrorism is not new. Biological agents have been used to spread disease deliberately since antiquity.[5] The use of fomites, cadavers, carcasses and other contagious items has occurred in order to contaminate, either directly or indirectly. Direct methods have involved the tossing of contaminated cadavers over city walls, while indirect methods have involved the contamination of water sources. Such documented uses of biological warfare and bioterrorism have stretch back to the ancient Greeks and as recently as the Second World War (Table 1).

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More recently, in 1992, the Aum Supreme Truth cult began cultivating anthrax with the aim of using it as a method of bioterrorism, in the belief that it would lead to a world war and subsequent world domination by the cult's founder, Shoko Asahara.[6] The following year, in July 1993, aerosolised anthrax spores were released in an area of Tokyo, but this failed to cause any cases of inhalation anthrax, probably for a number of reasons. Firstly, the cult unknowingly used the attenuated variety of anthrax (Sterne 34F2 strain), used in cattle vaccines, which possesses the plasmid-mediated toxin but not the capsule.[7] Secondly, the weather conditions were not favourable when aerosolisation occurred.[6] Similar use of botulinum by the cult also failed, again because it used an attenuated variety of the bacterium.

Such activities highlight the modern threat of bioterrorism, which went unnoticed at the time and were only investigated retrospectively (between 1999 and 2001), after the cult's sarin attack in 1995.[7]

The largest act of bioterrorism known to have occurred in the USA was the deliberate use of *Salmonella typhimurium* to contaminate food salad bars in restaurants in Oregon in 1984.[8,9] The act was performed by followers of the Bhagawan Shree Rajneesh cult, which attempted to affect local elections by keeping voters at home. The outbreak of salmonellosis that followed involved 751 individuals but the source was not confirmed by sufficient evidence until the following year.

In 1995, a near-successful attempt by individuals in the US to obtain the plague bacillus indicates the possible ease with which agents of bioterrorism can be obtained. This was attempted with only a credit card purchase and false

letterhead. The attempt was thwarted but the US authorities could only prosecute for mail fraud because there was no law against requests for such agents. Of course, this has now changed in the US.

In the UK, the Health and Safety Executive, Ministry of Agriculture, Food and Fisheries and the Department of the Environment and Transport, in combination, enforce the contained use, handling and deliberate release of pathogens, while other countries have similar arrangements. In 1996, intentional contamination of muffins and doughnuts with *Shigella dysenteriae* type 2, left in the tea room of a medical centre, led to severe gastrointestinal illness in 12 employees.[9] However, the source and motive for the outbreak have not been confirmed, although the bacterial strain is likely to have originated in the laboratory and the act performed by someone with access to the freezer and knowledge of basic microbiological technique.

The most recent act of bioterrorism involved letters that were tainted with anthrax and sent to various agencies, both civilian and federal, in October 2001.[10,11] Even though the Centers for Disease Control and Prevention (CDC) warned about the possibility of bioterrorism following the 11 September attacks, the authorities could not have been fully prepared for what happened.

The first and fatal case of inhalational anthrax was detected following routine hospital admission, based on clinical presentation and laboratory investigations. Further exposures occurred and envelopes containing spores of *Bacillus anthracis* sent through the US Postal Service to individuals in the media and to US senators were identified in New York and Washington DC. Hoaxes involving envelopes containing harmless white powder complicated investigations and led to increased hysteria among the public. By the middle of November there were seven cases of cutaneous anthrax and 11 cases of inhalational anthrax, five of which proved fatal.[12] An estimated 10 to 26 cases were prevented by prophylactic treatment given to over 30,000 individuals.[13,14]

An agreement to restrict the first use of biological weapons is contained in the 1925 Geneva Protocol. This did not, however, prevent agents being researched or prepared for defence purposes. President Richard Nixon renounced biological weapons due to their insignificance compared to American nuclear capabilities, and the world agreed to the control of biological weapons under the Biological and Toxins Weapons Convention (BWC), which came into force in 1975. By 1997, 140 nations had ratified the agreement.

The convention prohibits the acquisition of biological materials for hostile purposes but does not prohibit research into biodefence or enforce compliance. Interestingly, some 20 years later, the number of countries with a biological weapons capability has doubled.[15] In 1989, however, the US Congress passed the Biological Weapons Act to protect the US against bioterrorism. The Act defined as a federal crime any activity relating to the development, manufacture, transfer or possession of any "biological agent, toxin, or delivery system" for "use as a weapon".[16]

In addition, the CDC, Association of Public Health Laboratories, Federal Bureau of Investigation (FBI) and United States Army Medical Research Institute of Infectious Diseases established the National Bioterrorism Laboratory Response Network (NBLRN).[17] This US-wide system, initiated in 1999, was designed to link state and local public health laboratories with specialist laboratories. The NBLRN is a critical component of the CDC's mission and builds on a longstanding, nationwide system of public health laboratories.

Importantly, all states have access to laboratories with containment facilities for dealing with and identifying a number of bioterrorism agents. Although the introduction of the Biological Weapons Act did not prevent the anthrax attacks of late 2001, the member laboratories of the NBLRN were used to confirm the identity of anthrax isolates.

Toxins and bioterrorism

Biological warfare and bioterrorism posed two of the greatest threats to the military and civilian population of the USA, according to US President Bill Clinton.[18,19] He was not to know that just a few years later a major act of terrorism would kill thousands of civilians and spread fear worldwide. Although bioterrorism has been a threat for many decades, it is only recently that it has reached the forefront of the public's imagination.[20] However, recent events have posed new threats and imposed new tensions.[21] Due to the superior military power of the West, the use of biological warfare could become a reality. The current threat lies with terrorist organisations that possess biological weapons or have links with countries that have such weapons.

Biological weapons, like other types of weapon, can be evaluated on their effectiveness, method of delivery, cost and availability. They can fulfil all these criteria very well, although it is extremely difficult to reach the standard of a perfect biological weapon. The availability of certain bacteria or viruses to so-called rogue nations and terrorist organisations poses a real threat to mankind.

There are a number of agents that could be involved in a bioterrorist campaign[22,23] and the CDC has prepared a list of 41 agents or groups of agents (http://www.bt.cdc.gov). Many of these are responsible for serious diseases that have left an imprint on the history of mankind but have been under control in developed countries for many years. Several of these produce highly potent toxins for which treatments would be largely ineffective during an act of bioterrorism.

Although many toxins could be used as agents of bioterrorism, whether derived from animals, plants or microbes, it is the purpose of this review to describe those that are derived from bacteria (Table 2). As one would expect, these toxins are produced by major human pathogens such as *Clostridium botulinum*, the cause of botulism, and *B. anthracis*, the cause of anthrax.

The long-term issues of bioterrorism are also important. If a bioterrorist act, however small, were to take place, there is the potential for long-term contamination. Anthrax spores can survive in the environment for many decades and it would be virtually impossible to decontaminate an area targeted in this way and could render it inhospitable.[24,25]

Botulinum toxin

Botulism is a fairly uncommon disease of humans and animals but has a worldwide distribution.[26] It is caused by the production of a toxin of *C. botulinum*, an anaerobic Gram-positive spore-forming rod. The spores of *C. botulinum* may

Table 1. Examples of the historic uses of biological warfare.

Action	Who	Date	Reference
Wells used for drinking water were contaminated with corpses	Ancient Greeks	–	–
Plague-infested cadavers were thrown over city wall	Tatar force during siege of Kaffa	14th century	63
Smearing of pungi sticks with excrement	Viet Cong	Early 1960s	64
Smallpox	British forces against Native Americans during French and Indian War	18th century	5
Bacillus anthracis and *Burkholderia mallei* used to infect mules in order to infect French cavalry	Germans	1916	65
Food items contaminated with *B. anthracis*, *Yersinia pestis*, *Vibrio cholerae*, shigella and salmonella	Japanese bioattacks against 11 Chinese cities	Second World War	66

be found in the environment (eg the soil and the sediment of seas and lakes). As with many anaerobic Gram-positive bacterial spores, those of *C. botulinum* are heat resistant.

Botulism is potentially lethal and is usually due to foodborne intoxication. In infants, however, the disease may be due to intestinal infection with the organism and often occurs in those under 12 months old.[27] Infant botulism often occurs at the time of weaning, when the intestine's bacterial flora is changing.[28] Wound botulism, although very rare, can also occur following infection of a wound with *C. botulinum*. It is very similar to foodborne botulism but may include a fever due to the formation of an abscess at the site of the wound.

There are seven antigenically-different types of toxin produced by the various strains of *C. botulinum* and they are the most potent natural poisons known to man.[26,29,30] To put this in perspective, botulinum toxin is 15,000 times more toxic than VX nerve agent and 100,000 times more toxic than sarin.[31,32] A fatal dose for man has been estimated at 0.1–1.0 µg. Of the seven toxins (A–G), types A–F are responsible for human botulism, with A, B and E being the most common.[33]

Type A toxin is mostly associated with vegetables, type B with meat and type E with fish. The majority of cases are due to home-prepared foods and not commercially processed foods; therefore, most cases are isolated. Large outbreaks, due to the contamination commercially-processed foods, are rare but important. Pure botulinum toxin is a white crystalline substance that dissolves readily in water but rapidly becomes inactive after exposure to the air.

The toxins are best produced in anaerobic conditions at 30°C, are heat labile and are destroyed by oxidation. They are also immunogenic and can be transformed into toxoids (ie they can be used as vaccines). The toxins are absorbed by the gastric and upper intestinal mucosa, where it interferes with neurotransmission at peripheral cholinergic synapses by binding irreversibly to the presynaptic nerve surfaces of neuromuscular junctions. After internalisation, blockage of acetylcholine release occurs.

The incubation period of foodborne botulism is between six hours and 16 days (mean: two/three days). The initial stage of the disease is short and is often characterised by nausea and vomiting, followed by bilateral and symmetrical paralytic ocular manifestations. As the disease progresses, the patient remains afebrile with normal consciousness but has difficulty in swallowing, difficulty in speaking and often has double vision. Persistent constipation is common and urinary tract disorders such as dysuria and retention may cause further problems. Respiratory problems due to paralysis are less frequent but may be serious if they do occur. Secondary bacterial infections may create problems and artificial ventilation may also be necessary.

Mortality rate in foodborne botulism is 10–50%. Many patients recover without sequelae, although this can takes weeks or months, depending on the intake of toxin, due to the time it takes for new nerve terminals to grow. Infant botulism is characterised by an acute flaccid paralysis that begins in the muscles of the head, face and throat, and then extends symmetrically to involve the trunk and extremities. The mortality is low (approximately 2%) if the infant is hospitalised.

The low number of cases does not warrant a national vaccination programme but the presence of spores in the environment means that eradication is not possible. Early vaccines were developed in the 1940s but improved vaccines have since been introduced.[34] Outbreaks from commercially-processed foods are decreasing in frequency due to improved production methods, but this form of delivery in a bioterrorism event is likely and thus awareness of the incidence and implications of botulism remains necessary.[35,36] Such measures include the establishment of appropriate surveillance systems.[37]

Enterotoxin B

Staphylococcal enterotoxin B (SEB) is one of a number of enterotoxins produced by certain strains of *Staphylococcus aureus*.[38] Not all toxins are lethal but they may result in significant morbidity. The SEB toxin is the most common cause of classic food poisoning and has been studied as a potential biological agent of war, as it is easily aerosolised, is very stable and can cause widespread systemic damage, multiorgan system failure, and even shock and death when inhaled at very high dosages. However, SEB is classified as an incapacitating agent because, in most cases, aerosol exposure results not in death but in a temporary, profoundly incapacitating illness lasting up to two weeks. Clearly this would be devastating if used in an act of bioterrorism.

Staphylococcal enterotoxin B consists of 239 amino acid residues and has a molecular weight of 28 kDa.[39,40] It is a relatively stable compound that is easily soluble in water. It is very resistant to temperature fluctuations and can withstand boiling for several minutes. In a freeze-dried state, SEB can be stored for more than a year. For aerosol exposure, the effective dose – one capable of incapacitating 50% of the

Table 2. Examples of bacteria and their toxins which have potential use in bioterrorism.

Bacterium	Disease	Toxin	Mode of action
Bacillus anthracis	Anthrax	Anthrax toxin	Adenylate cyclase activity which increases cellular cyclic AMP levels
Clostridium botulinum	Botulism	Botulinum toxin	Inhibits release of the stimulatory neurotransmitter acetylcholine
Clostridium perfringens	Food poisoning and gas gangrene	Perfringens toxins	Varies according to toxin
Staphylococcus aureus	Food poisoning and multi-organ system failure	Enterotoxin B	Crosslinks major histocompatibility complex (MHC) class II proteins, leading to the release of cytokines and T-cell proliferation

exposed human population (ED50) – is 0.0004 μg/kg, and the lethal dose (LD50) is 0.02 μg/kg. The SEB toxin is produced and excreted in foodstuffs that have been improperly refrigerated, stored or handled, and ingestion causes food poisoning.[41,42]

The incubation period is between one and eight hours. Classic symptoms are an abrupt onset of intense nausea, vomiting, cramping abdominal pain and diarrhea, which incapacitate the patient. Most cases are self-limiting and resolve in eight to 24 hours. The onset of symptoms after inhaling SEB may vary from one to six hours. Sudden onset of headache, fever, myalgia, non-productive cough, chills and shortness of breath can be caused by inhalation of low-dose SEB. Fever may last two to five days, and cough may persist for up to four weeks. Higher exposure to SEB may lead to septic shock and death.

Staphylococcal enterotoxin B is commonly referred to as a bacterial superantigen.[43,44] After aerosol exposure, it is an extremely potent activator of T cells because it binds directly to the major histocompatability complex class II proteins on target cells.[45] This also stimulates the production and secretion of various cytokines. Therefore, many of the effects of SEB are mediated by the host's immune system.

In contrast, ingestion of SEB produces profound gastrointestinal symptoms including anorexia, nausea, vomiting, and diarrhea, which are believed to be mediated through the release of histamine and leucotrienes from mast cells.

Anthrax toxin

Anthrax has been known to cause disease in animals and humans for hundreds of years. It is a Gram-positive bacillus that is facultatively anaerobic, produces a polypeptide capsule *in vivo*, and is also able to enter a spore state. There are two virulence factors of *B. anthracis* (polypeptide capsule[46] and toxin[47]), both of which are encoded by genes on separate plasmids.[48] If one of either plasmid is not present in a strain of *B. anthracis* then the bacterium is less virulent. The toxin has three factors, the oedema factor, the protective antigen and the lethal factor. The overall effect of the toxin is to depress the cerebral cortex, resulting in respiratory distress, cardiac collapse, shock and finally death.

Anthrax is primarily a disease of herbivores, which usually acquire the bacterium by ingesting contaminated foodstuffs such as feed, grass and water or by ingesting spores present in soil. Anthrax spores are able to survive in the environment for prolonged periods of time. Water and soil can become further contaminated by haemorraghic effusions from an animal dying of anthrax.

Human anthrax is almost always acquired by exposure to an infected animal, whether dead or alive. There are three forms of human disease: cutaneous, intestinal and pulmonary. Cutaneous anthrax occurs after infection of a cut, lesion or bite on the skin.[49–52] Symptoms occur two to three days after infection and consist initially of a small boil-like papule, followed by the development of a ring of vesicles around the original papule. The papule ulcerates to form an eschar (a hard plaque covering an ulcer) which enlarges, turns black and results in localised oedema. This eschar covers the surrounding vesicles so that the lesion is usually approximately two centimetres in diameter, although it may be much larger (≥ 10 cm) in some cases. If uncomplicated, the anthrax bacilli remain localised. Adenitis is common but fever is mild or absent. The eschar begins to resolve after 10 days, although total resolution can take two to six weeks.

The main complications of cutaneous anthrax are meningitis,[53] septicaemia and secondary bacterial infection. Meningitis occurs in approximately 5% of cutaneous anthrax cases. Intestinal anthrax occurs between two to five days after the ingestion of anthrax spores from infected meat or milk.[54] The eschar, as described for cutaneous anthrax, often occurs in the terminal ileum or caecum, although it may occur at other sites along the gastrointestinal tract. Symptoms include nausea, vomiting, anorexia, fever, abdominal pain and bloody diarrhoea. Pulmonary (inhalation) anthrax is the most severe form of anthrax, and symptoms of disease occur two to five days after infection.[55] Initial symptoms include mild fever, fatigue and malaise. After the mild initial phase ends, an acute illness develops in which the patient may first vomit or cough blood, and than suffer dyspnoea, cyanosis, severe pyrexia and disorientation. This acute illness is followed rapidly by coma and is invariably fatal.

Owing to the devastation it could cause, anthrax has maintained its position as one of the leading bacteria for use as a biological weapon. Pulmonary anthrax, however, is difficult to induce and would require inhalation of an estimated 50,000 spores per individual. However, casualties would be numerous.

Clostridium perfringens toxins

Clostridium perfringens is a common anaerobic bacterium associated with three distinct disease syndromes: gas

gangrene, clostridial food poisoning and enteritis necroticans.[56,57]. Each syndrome requires specific conditions to induce disease. It is therefore difficult to cause disease by spreading the organism in the environment. However, the bacterium elaborates at least 12 protein toxins, and one or more of these could be produced, concentrated and used as a weapon. Waterborne disease is conceivable but unlikely, while the alpha toxin would be lethal if acquired by aerosol. Other toxins from the organism might be co-weaponised to enhance effectiveness. For example, the epsilon toxin is neurotoxic in laboratory animals.

Gas gangrene is a well-recognised life-threatening emergency.[57] Symptoms may be subtle before fulminant toxaemia develops, and the diagnosis is often made at post-mortem examination. The bacteria produce toxins that are responsible for the high mortality associated with clostridial myonecrosis, and which produce the characteristic intense pain that is out of proportion to the size of the wound.

Signs of systemic toxicity (confusion, tachycardia and sweating) appear within hours. Most *Clostridia* species produce large amounts of carbon dioxide and hydrogen, which cause intense swelling and give rise to the term 'gas' gangrene. Clinical features include necrosis, dark red serous fluid, and numerous gas-filled vesicles. The infection may progress rapidly, and early diagnosis and therapy are essential to prevent rapid progression to toxaemia and death. Pulmonary findings may lead initially to diagnostic confusion with SEB poisoning. Liver damage, haemolytic anaemia and thrombocytopenia are not associated with SEB and the pulmonary findings should be reversible in SEB.

Clostridial food poisoning is characterised by intense abdominal cramps and diarrhoea, which begin eight hours to one day after consumption of foods containing large numbers of *C. perfringens*. The illness is self-limiting, usually within 24 hours, but less severe symptoms may persist for one to two weeks. Only a few deaths have been reported as a result of dehydration or other complications. The more serious, but rare, enteritis necroticans is caused by ingesting food contaminated with type C strains. It begins as a result of ingesting large numbers of *C. perfringens* but pogresses to intestinal necrosis and septicemia.

How would toxins be used?

As with many other agents, toxins are usually prone to degradation from desiccation and temperature. While the toxin must be easy to produce, be highly effective after ingestion and be stable, the delivery method is paramount in the use of toxins in bioterrorism. Cruise missiles have been suggested as the ideal delivery system because they lay down a cloud close to the ground, at an altitude of approximately 100 metres, and their subsonic speed avoids overheating.[58] However, this method of delivery is only ideal if the bioterrorism agent is to be inhaled as small particles or intended to infect soil. Moreover, the terrorist would require finance and access to such technology.

Arguably, the most effective delivery method for a toxin is in water, which is used for many everyday tasks such as irrigation, manufacturing and consumption. Although modern water treatment plants are highly effective in removing many toxic agents, treatment plant failures could result in bacteria, parasites and toxins passing into public water supplies.

However, bioterrorism also places a new light on foodborne disease. Basic sanitation is already important in food preparation but this can also help minimise the occurrence and spread of bioterrorism agents. Importantly, foods should only be purchased from reputable vendors and packaging should be intact. Food-associated bioterrorism, as well as accidental contamination of foods, provide examples of how large numbers of individuals could be affected.

Biotechnology has led to the rapid emergence of genetic manipulation. Although this technology has had a profound and positive effect on medicine, it could be used for other purposes. Modern genetic methods could lead to the modification of toxins so that they bind more effectively to the target cell or so that they could be delivered in a regulated format. The latter could target defined populations or rely on a host response before toxin activation. Genetic manipulation requires fairly specialised laboratory facilities so the likelihood of this is reduced unless sponsored, for example, by a state that supports terrorism.

Economic effects of bioterrorism

Bioterrorism is often politically or religiously motivated and can have profound political, religious, economic and social effects. However, it does not have to be directed against human beings to have an effect on world economies or human health. Although the most deadly and likely agents to be used are anthrax, plague, smallpox and botulism, other biological agents could be used that would have major economic consequences. The impact of a bioterrorist attack with anthrax on Washington DC, for example, has been estimated at $26.2 million per 100,000 people exposed.[59] Immense difficulties would occur for local, regional and national officials in coping with a major attack, not only in identifying early cases of disease but also in the coordination of resources.

However, the four most deadly agents require specialist knowledge and expertise for delivery and do not, therefore, provide the most obvious channel for terrorists with limited resources. In addition, the aim of terrorists is to cause disruption rather than death. Any terrorism event results in a temporary or permanent economic effect, whether it be due to reduced tourism, air travel or finances. Major human and economic disruption, therefore, can be caused by the use of biological agents.

Animals or crops, which represent a high percentage of domestic product, could be targeted.[60–62] Outbreaks of animal disease could, in theory, be started by bioterrorism, resulting in animal loss, decreased tourism revenues and large compensation costs. Foods could also be targeted using bacteria such as salmonella, shigella and cholera. Even influenza and cryptosporidia could be used as biological weapons, the main aim being to cause disruption and terror in the community.

Conclusions

Bioterrorism is now a reality in an era in which political and religious agendas have changed. Bacterial toxins

could be used during a bioterrorism event, although they may account for only a proportion of all biological agents that could be used. However, bacterial toxins are the cause of some of the major human infectious diseases. Many countries have taken a major stance against terrorism, including bioterrorism, in an attempt to counteract such activities. Events during the last decade have taught us that bioterrorism events, however small, can have major repercussions and that the public should remain vigilant. □

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