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Biological warfare agents

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ABSTRACT

Various types of biological weapons have been known and practiced throughout history, including the use of biological agents such as microbes and plants, as well as biotoxins and the venoms that can be derived from them. In ancient civilisations, the attempt was to infect and kill enemies by throwing cadavers into water wells. Emperor Barbarossa during the battle of the Italian town, Tortona, in 1155, did the same. In modern times, America and the Soviet Union also undertook biological warfare and anti-biological warfare protection activities. This even intensified after WWII. When the Soviet forces captured and interrogated some Japanese scientists in 1945, they utilized the obtained information in their own biowarfare program and their research accelerated in 1946. Following this, a series of new biowarfare study centres and production facilities was constructed in the 1950s. The Soviet biowarfare program included tularemia, anthrax, brucellosis, plague, glanders, marburg virus, smallpox virus, and VEE virus. During the time of the Korean War, it was believed that biowarfare agents were used by America against Soviet Union. The Americans had began their own program in Fort Detrick (former Camp Detrick) in 1943 and a new production facility at Pine Bluff Arsenal in Arkansas was constructed. The United States of America started producing tons of *Brucella suis* in 1954. In the peak year of their program, they involved about 3,400 people and a number of agents: *Bacillus anthracis*, *Francisella tularensis*, *Brucella suis*, *Coxiella burnetti*, *Venezuelan equine encephalitis virus*, yellow fever, botulin, *Staphylococcal enterotoxin*, and the anti-crop agents *Pyricularia oryzae* and *Puccinia graminis*. Due to public pressure, President Nixon declared a unilateral halt in 1969 to biological weapon projects. The only permitted research was defensive, such as diagnostic, vaccines, and chemotherapies tests – as evidenced in the UK where the base in Porton Down was converted into a defence institution.

Keywords: *Coxiella burnetti*, *Brucella* species, *Burkholderia mallei* and *pseudomallei*, Alphaviruses, Toxins, *Rickettsia prowazekii*, *Chlamydia psittaci*, *Salmonella* species, *Shigella dysenteriae*, *Escherichia coli*, *Cryptosporidium parvum*, *Vibrio cholerae*

1. INTRODUCTION

Various types of biological weapons have been known and practiced throughout history, including the use of biological agents such as microbes and plants as well as biotoxins and the venoms which can be derived from them. In ancient civilisation, the attempt to infect and kill enemies by throwing cadavers into water wells made by Emperor Barbarossa during the battle of the Italian town, Tortona, in 1155 [1]. Another strategy used by Mongol armies in 1346 was to hurl plague-infected cadavers into the besieged Crimean city of Caffa transmitting the disease to the inhabitants and the fleeing survivors of the siege spread the plague from Caffa to the Mediterranean Basin [2]. In 1495, the Spanish offered wine spiked with the blood of leprosy patients to the French near Naples [3]. In 1797, around the plains of Mantua, Italy suffered floods reportedly spread by Napoleon to enhance the spread of malaria [1].

In the late 19th century, scientists introduced the concept of microorganisms as agents of infectious diseases. Germany was suspected to be the first one to use weapons of mass destruction and sabotage during World War I (WWI), both biological and chemical where they employed cholera, anthrax and plague. This kind of sabotage was carried out in the USA, Romania, France and Spain, and later in Argentina and Norway [4, 5]. Due to the exploitation of chemical weapons in WWI and understanding of biowarfare weapon possibilities used by Germany, they were prohibited from storing, and importing or using many types of weapons according to Treaty of Versailles. This move led to the formation of the Geneva Protocol: “Protocol for the prohibition of the use in war of asphyxiating, poisonous or other gases, and of bacteriological methods of warfare” in 1925 and entered into force in February 1928. This protocol aimed to prohibit the use of poisoned weapons.

However, although these protocols, including the past treaties, were all agreed to by the League of Nations, did not guarantee a means of control, and thus failed to prevent interested parties from developing and using biological weapons [4].

Japan and United States of America (USA) did not ratify the Geneva protocol. Japan started their modern biological arm race in 1932 until the end of World War II (WWII) in which more than 10,000 prisoners were believed to have died as a result of experimental infection during the Japanese program [3]. France ran a similar program in 1936, Canada in 1939 and the United Kingdom (UK) in 1940 [6]. The British secretly developed their own biological warfare program in Porton Down focused on brucellosis, tularemia, venezuelan equine encephalomyelitis (VEE) and vaccinia viruses. Their practical experiments were realized on Gruinard Island near the coast of Scotland. The island remained contaminated until 1986 and successful decontamination was accomplished using formaldehyde [7]. The German effort for obtaining biological weapons was minimal during WW II [5, 8].

USA and Soviet Union has continued their protection activities and was even intensified after WWII. When the Soviet forces captured and interrogated some Japanese members in 1945, they utilized the obtained information in their own biowarfare program and their activities accelerated in 1946. Following this, a series of new biowarfare research and production facilities was constructed in the 1950s. The Soviet biowarfare program included tularemia, anthrax, brucellosis, plague, glanders, marburg virus, smallpox virus, and VEE virus [9]. During the time of the Korean War, it was believed that biowarfare agents were used by the USA against Soviet Union. The USA began their own program in Fort Detrick (former Camp Detrick) in 1943 and a new production facility at Pine Bluff Arsenal in Arkansas was made. USA started producing tons of *Brucella suis* in 1954. In the highest peak

of their program, they involved about 3,400 people and a number of agents like *Bacillus anthracis*, *Francisella tularensis*, *Brucella suis*, *Coxiella burnetti*, *Venezuelan equine encephalitis virus*, yellow fever, botulin, *Staphylococcal enterotoxin*, and the anti-crop agents *Pyricularia oryzae* and *Puccinia graminis* [5]. Due to public pressure, the late President Nixon declared disarmament in 1969 to stop biological weapon projects. The only permitted research was defensive, such as diagnostic, vaccines, and chemotherapies tests just like UK where the base in Porton Down was converted into a defence institution.

The most important dates in biological weapons history was in the year 1972 when member nations ratified the biological and toxin weapons convention, that entered into force in March 1975: “The United Nations Convention on the prohibition of the development, production, and stockpiling of bacteriological and toxin weapons and their destruction” [10]. The convention tackled the prohibition of biological weapons after 1975 but the reality was different since Soviet Union continued its’ program and taking advantage of the rapid progress in microbiology and biotechnology that led to the formation of special secret organizations, which was named Biopreparat, to develop biowarfare technology and agents. They were accused of supplying mycotoxins to its’ Vietnamese and Laotian communist allies for military use against resistance forces in Laos and Cambodia, and of using the same agents in combat operations in Afghanistan in the 1980s [5]. In parallel, Iraq was one of the countries that successfully built industrial biological weapons which was included in their three weapons of mass destruction, i.e nuclear, chemical and biological. Their program in biowarfare started in 1975. They explored and investigated Botulinum toxin, *Bacillus anthracis* and *Clostridium perfringens* spores, camelpox virus and ricin but their sites were then eventually destroyed during the gulf war [11]. South Africa also initiated a biowarfare program in 1980, and used anthrax for individual assassinations and cholera for contaminating water supplies during attacks against freedom fighters [5].

Acts of bioterrorism have not been controlled in the last decades. In September 1984, Oregon experienced America’s first community bioterrorism attack led by the followers of Bhagwan Shree Rajneesh, who established a commune in the county and intentionally infected restaurant diners in The Dalles as part of a plot to take over county government and at least 750 people became ill with a unique strain of Salmonella (<https://www.nwpublichealth.org>). In Tokyo, Japan, the attempt to disseminate anthrax in 1993 by the Aum Shrinikyo cult was not successful but the cult was able to recruit many professionals, including those with scientific and medical training and were able to obtain *Bacillus anthracis* through their contacts. Nobody was harmed by the anthrax attack because the source was a non-pathogenic strain and the authorities were not even aware of the release until later when the cult was investigated for the release of Sarin gas on the Tokyo underground [12].

Recently, the threat of bioterrorism attacks has attracted attention once again and threatens the whole world due to the recent chemical attack that has struck Syria [13] which killed hundreds of men, women, and children as well as the *Bacillus anthracis* sporecontaining letter attack [14, 15] that happened in United States shortly after the 9/11 attack. The presented history on biological warfare and bioterroristic attacks would highlights the risks associated with biowarfare agents, and how biowarfare could be used for mass destruction in the future, and the associated threats that could bring to humankind.

Considering the general availability of know-how to culture microorganisms in large quantities, there is now a global argument about the possibility of using different pathogens

with high risk not only limited to public health safety but also to plants and animals for bioterrorism attacks. There are numerous pathogens, including bacteria, viruses, fungi, toxins among others, which are listed by various agencies as potentially dangerous agents [16].

Critical biological agents based on several criteria have been classified in three categories by the Centers for Disease Control and Prevention (<http://www.bt.cdc.gov/agent/agentlistcategory.asp>). Agents that cause greatest harm are classified as category A and include *Bacillus anthracis*, *Yersinia pestis*, *Variola major*, *Francisella tularensis*, and viral hemorrhagic fevers. These agents pose a high risk to national security because they can be easily disseminated or transmitted from person to person or potential delivery through weapons which result in high mortality and severe impact on human health, causing public disruption and panic. Category B includes agents that are moderately easy to disseminate, and which result in moderate morbidity rates and lower mortality rates than agents in category A. Agents in this category included *Coxiella burnetii*, *Brucella* species, *Burkholderia mallei* and *pseudomallei*, Alphaviruses, Toxins, *Rickettsia prowazekii*, *Chlamydia psittaci*, *Salmonella* species, *Shigella dysenteriae*, *Escherichia coli*, *Cryptosporidium parvum*, and *Vibrio cholerae*. Category C includes emerging pathogens that are readily available and easily disseminated such as Nipah virus, Hentavirus, Tickborne hemorrhagic fever viruses, Tickborne Encephalitis virus, Yellow Fever, and multidrug-resistant tuberculosis [17]. Although category C is considered as the lowest risk among the three categories, agents that belong to this category should not be neglected as they are also considered to have potential for high morbidity, mortality rates and major health impact. The following section discusses in details some important high risk pathogens.

1. 1. *Bacillus anthracis*

Anthrax is an acute infectious zoonotic disease caused by the spore-forming, aerobic, Gram positive, non-motile bacterium, *Bacillus anthracis*. The bacteria exists in the environment as a spore and can remain viable in the soil for decades [18]. Anthrax was a major cause of death for animals all over the planet until the end of the 19th century, with occasional, sometimes extensive, contamination to humans [19]. Spores that have been ingested by herbivorous animals can germinate inside the animal to produce the virulent vegetative forms that replicate and eventually kill the host. Products from infected animals or exposure to dead animals serve as a reservoir for human infections [20].

There are three major clinical forms of anthrax that affect humans; cutaneous, gastrointestinal and inhalational anthrax. Among the three, cutaneous anthrax is globally the most prevalent naturally occurring anthrax infection. This results when any broken skin is exposed to the spores that form ulcer and black eschar. Fever can also occur during the incubation period. Gastrointestinal anthrax is typically related to ingestion of spore contaminated meat and there are two forms of gastrointestinal anthrax: oropharyngeal and intestinal. Spores settle in the pharyngeal area and produce ulcers in oropharyngeal anthrax. The mean incubation of the spores is about 42 hours. In intestinal anthrax, spores are deposited and cause ulcerative lesions anywhere from the jejunum to the cecum. A patient frequently suffers from nonspecific gastrointestinal symptoms, fever and neck swelling. The last form is inhalation or pulmonary anthrax following inhalation of thousands of spores. The first symptoms are similar to influenza and after 2 or 3 days of high fever with haemorrhage there is a rise in systematic infection. Gastrointestinal and inhalation anthrax are fatal when left untreated and undiagnosed and immediate treatment with antibiotics should be employed.

Biological and chemical techniques have been considered in the last decades to be useful in identification and detection of anthrax spores. Identification of *Bacillus anthracis* has been found to be difficult because of its similarity with other strains in its genus. Polymerase chain reaction (PCR) and immunoassays are the two most employed biological methods to detect anthrax spores. PCR-based assays can accurately differentiate pathogenic *Bacillus anthracis* strains from a pathogenic *Bacillus anthracis* from non-anthraxis *Bacillus* species [21] while immunoassay is one of the most currently used methods in clinical diagnosis [22]. Recently, specific detection and accurate identification of the presence of *Bacillus anthracis* in any media including foods has been determined by the use of pyrosequencing technology [23].

1. 2. *Brucella* species

Brucellosis is a widespread zoonotic disease caused by *Brucella* spp. affecting both humans and animals [24]. Human brucellosis remains the most common zoonotic disease worldwide [25]. Domestic animals like cattle, sheep, goats, swine and even dogs, especially sheppard dogs are the natural reservoirs of the organisms. Humans get infected through conjunctiva or skin abrasions when exposed to animal fluids infected with the disease, through ingestion and inhalation [26]. After infecting the host, the pathogen becomes sequestered within cells of the reticuloendothelial system, the mechanism by which brucella enters cells and evades intracellular killing, degrading host's immune system [24]. Brucellosis in human beings is rarely fatal but it can be severely debilitating and disabling. It is a multisystemic disease with a broad spectrum of symptoms, although it can be asymptomatic as well. It begins as a flu-like disease with symptoms such as fever and generalized aches. Gastrointestinal signs, i.e. anorexia, nausea, vomiting, diarrhea, and constipation, coughing, and pleuritic chest pain can also be seen. The most common complications are arthritis, spondylitis, epididymo-orchitis, and chronic fatigue. Endocarditis is one of the most serious complications of brucellosis. Some other organs are also affected, resulting in lymphadenopathy, deep vein thrombosis, granulomatous hepatitis, osteomyelitis, anemia, thrombocytopenia, and nephritis [25].

Five species have been recognized in the past, according to relative animal host specificity [27] and additional 5 more species has just been recently added [26]. Pathogenicity of five *Brucella* species for humans has been confirmed. *Brucella melitensis* was isolated in 1887 in Malta (hence called Malta fever) by David Bruce from the spleen of a soldier who died from acute brucellosis. It usually affects sheep and goats whilst *Brucella abortus* causes abortions in cattle. *Brucella suis*, which was also isolated from wild hares, causes the disease mainly in swine which is also pathogenic for humans. *Brucella canis* isolated from dogs, could be also pathogenic to humans. Finally, *Brucella marina* is found in sea mammals (whales, seals) in the Atlantic Ocean [26,27]. The disease in humans is mainly due to *Brucella melitensis* as the most pathogenic species followed by *Brucella suis*, while *Brucella abortus* is considered as the mildest type of brucellosis.

Serological and cell culture techniques are the usual diagnostic methods used for both animals and humans. The Wright test or agglutination reaction is still considered the standard method [27] and in recent years, methods of molecular biology have been used increasingly often in the diagnostics of brucellosis, particularly PCR [26].

1. 3. *Francisella tularensis*

Tularemia, also known as rabbit fever, is a highly infectious zoonotic disease caused by the non-motile, non-spore-forming, Gram-negative coccoid rod bacterium, *Francisella tularensis*. It occurs naturally in lagomorphs (rabbits and hares), but many animals have been reported to be infected. Transmission to humans is mostly associated with inhalation of aerosolised bacteria, handling of infected animals, arthropod bites, and ingestion of contaminated foods and water [28, 29]. The usual incubation period is 3-5 days but symptoms can become visible between 1 and 21 days depending on the route of infection. Clinical manifestation of the disease in humans can occur in different forms ranging from skin ulcers to more severe forms such as life threatening-pneumonia [30].

Despite the fact that most of tularemia infections can be treated with antibiotics [31], it is still considered as life-threatening due to its high virulence, transmission and mortality [32]. Identification of *Francisella tularensis* has been achieved using cultivation and molecular techniques including PCR [33] and real time PCR assays [34-36]. Besides the detection of the bacterial cell, the detection of specific antibodies in serum is the most widely used serological analysis technique for routine laboratory diagnosis of tularemia [37]. Enzyme-linked immunosorbent assay (ELISA) [38].

Western blot and other immunological assays can be used to detect seroconversion in patients. However, antibodies only appear 2 weeks or more after infection [39].

1. 4. *Yersinia pestis*

It has been believed that the bacterium *Yersinia pestis*, a nonmotile and slowly growing Gram-negative coccobacillus from the family Enterobacteriaceae, is considered the most likely cause of the most devastating disease outbreaks in human history; the Plague of Justinian and black death in the middle ages [40, 41]. Although some authors debate that the Plague of Justinian was caused by a different pathogen [42]. The natural reservoir of the plague foci are usually rodents that successfully integrate into the host's innate immunity and then propagates to induce bacteremia that is needed in order to constantly circulate and this is then transmitted by infected fleas to a new host through bites [43, 44] that result in the bubonic plague. In this disease, the organisms arrive in lymph nodes and multiply there, after being introduced by the bite of infected fleas. When bubonic plague is left untreated, it progresses to septicemic plague with increasing mortality that may result into pneumonic plague. Aside from the fleas, *Yersinia pestis* infection can also be transmitted by aerosols or contaminated food [45]. Following exposure to the agent, the incubation period takes from 2 to 6 days to appear with some symptoms like fever, malaise, nausea, vomiting and diarrhoea. The flu-like illness rapidly changes into bloody sputum within a very short period between 1 to 3 days after exposure to the agent. Treatment of human plague can be achieved and has been successful using antibiotics like streptomycin, gentamicin, doxycycline, and ciprofloxacin [43].

Several techniques have been developed for efficient detection of *Yersinia pestis*, including molecular techniques including PCR, biosensors, and immunoassay techniques [46]. Although *Yersinia pestis* is unstable in aerosol for longer times which impedes utilisation of this agent as a biowarfare, The CDC enlisted it into category A due to the high mortality and high virulence and resistance to its' environment as it can live for a long period of time in its' dead host, soil and in water.

1. 5. *Coxiella burnetti*

Coxiella burnetti is an intracellular, Gram-negative pathogenic bacterium which is the causative agent of Q fever (query fever) [47]. It is a zoonotic infection that manifests in humans primarily as an acute flu-like syndrome with potential complications including pneumonia and hepatitis. These signs and symptoms of human Q fever complicate and delay clinical diagnosis. The incubation period varies from a few days to weeks depending on the dose of bacteria and the immune system of the host [48].

The first outbreak of this disease was in Queensland, Australia in 1935. Infection typically occurs by inhalation of the bacterium contained in contaminated dust particles. Sources include barnyards and facilities housing *Coxiella burnetti* research programs. Some rare cases of Q fever have been reported in which a patient has been infected without direct contact with farm animals where the patient came down with Q fever-like symptoms after painting the walls of a science laboratory where a newborn lamb had been dissected. In addition, indirect accidental exposures have occurred with workers in offices near elevators used to transport pregnant sheep that were unknowingly infected with *Coxiella burnetti*. Due to its hazardous consequence, occupational hazards associated with research facilities has largely been eliminated due to implementation of modern biosafety equipment and protocols [49].

Confirmation of the disease normally involves testing for the presence of *Coxiella burnetti* - specific antibodies, which develop in patients 1–2 weeks after infection. The gold standard serological test for Q fever is an indirect immunofluorescence assay (IFA) that relies on serum reactivity. PCR-based technology is sensitive mainly in the early disease state but as the disease progresses the test sensitivity decrease [49]. The use of mass spectrometry analysis has also been employed for direct detection and identification of the bacterial cells [50].

1. 6. *Burkholderia mallei* and *pseudomallei*

Burkholderia mallei and *Burkholderia pseudomallei* are facultative intracellular, Gramnegative pathogens and the causative agents of glanders and melioidosis respectively [51], which are highly infectious via the respiratory route, and can cause severe diseases in humans and animals [52].

Glanders is a highly contagious and often fatal zoonotic disease primarily of solipeds such as horses, mules, and donkeys. Over the last 100 years, the occurrence of glanders has decreased due to the reduced economic reliance of using solipeds in terms of transportation. Eventhough glanders has almost been eradicated in most parts of the world, it is still considered as a life-threatening disease agent due to its high mortality. *Burkholderia mallei* was one of the first biological warfare agents used during WWI. Glanders can be transmitted through contact with abraded or lacerated skin, inhalation by bacterial invasion of the nasal, oral, and conjunctival mucous membranes. Depending on the route of infection, symptoms can vary from pulmonary, septicemic, or multitissue infection. The general symptoms can be low-grade fever, malaise, fatigue, headache, lymphadenopathy, and chest pain [53].

Melioidosis occurs following exposure to contaminated water or soil, usually through cuts in the skin or via inhalation [54]. *Burkholderia pseudomallei* is commonly found in soil and water in Southeast Asia and Northern Australia. The increasing cases of melioidosis are a serious global threat and clinical manifestations of melioidosis are extremely diverse.

Depending on the route of infection, symptoms vary from acute sepsis to chronic localised pathology to latent infections which can reactivate decades later. The lung is the most commonly affected organ when the bacterium is inhaled resulting in cough and fever that when left untreated and undiagnosed could lead to pneumonia, or secondary to septicaemic spread. The overall mortality rate in individuals infected with *B. pseudomallei* range from 30-70% resulting in this agent being categorised as one of the biological warfare agents [55]. Detection and identification of both species can be achieved through molecular recognition techniques such as PCR aside from the conventional way of cultures [56].

1. 7. *Bacillus thuringiensis* subsp. *kurstaki*

Bacillus thuringiensis subsp. *kurstaki* is a rod shape and Gram-positive bacterium that produces parasporal crystals during sporulation that are commonly found in soil and plants. It is used as a biological insecticide to control crop-damaging moths and *Lymantria dispar*. The gypsy moth is a major forest pest that is especially predominant along the eastern seaboard and in the Midwestern USA [57]. Although *Bacillus thuringiensis* subspecies are neither toxic nor pathogenic to mammals, including humans, some cases in animal experimentation has shown that intraperitoneal injection of *Bacillus thuringiensis* can cause death in guinea pigs and that pulmonary infection can result in the deaths of immunocompromised mice [58]. Reports of human disease are uncommon, however, several cases has been reported. An 18-year-old farmer developed corneal cancer after being accidentally splashed with a commercial *Bacillus thuringiensis* product into his eye [59]. Another case was a multiple thigh and knee abscess containing *Bacillus thuringiensis* found in a previously healthy soldier who was severely wounded by a landmine explosion in 1995 [60]. In another case study, it was found that *Bacillus thuringiensis* has been involved in an outbreak of gastroenteritis in four persons [61].

2. CONCLUSIONS

Various types of biological weapons have been known and practiced throughout history, including the use of biological agents such as microbes and plants as well as biotoxins and the venoms which can be derived from them. In ancient civilisation, the attempt to infect and kill enemies by throwing cadavers into water wells made by Emperor Barbarossa during the battle of the Italian town, Tortona, in 1155. USA and Soviet Union has continued their protection activities and was even intensified after WWII. When the Soviet forces captured and interrogated some Japanese members in 1945, they utilized the obtained information in their own biowarfare program and their activities accelerated in 1946. Following this, a series of new biowarfare research and production facilities was constructed in the 1950s. The Soviet biowarfare program included tularemia, anthrax, brucellosis, plague, glanders, marburg virus, smallpox virus, and VEE virus. During the time of the Korean War, it was believed that biowarfare agents were used by the USA against Soviet Union. The USA began their own program in Fort Detrick (former Camp Detrick) in 1943 and a new production facility at Pine Bluff Arsenal in Arkansas was made. USA started producing tons of *Brucella suis* in 1954.

Considering the general availability of know-how to culture microorganisms in large quantities, there is now a global argument about the possibility of using different pathogens with high risk not only limited to public health safety but also to plants and animals for

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