REVIEW ARTICLE

Anthrax and Bioterrorism

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Abstract

Bioterrorism is the deliberate discharge or dissemination of viruses, bacteria, or other germs used to cause sickness or death in people, animals, or plants. Though these agents are found in nature, they are usually modified to increase their ability to cause disease, make them resistant to current antibiotics, or to increase their ability to be spread into the environment. Biological agents can be spread through the air, through water, or in food. Terrorists may use biological agents because they can be extremely difficult to detect and do not cause illness for several hours to several days. Among these biological agents, Anthrax is considered to be the Category-A agent because of its high potency for adverse public health impact and serious effect on large-scale dissemination. Though there is a big hue and cry in the recent past about bioterrorism particularly after 9/11 attack, the history of bioterrorism is fairly old. In the present paper, we have sketched the history and the evolution of bioterrorism with reference to anthrax, particularly microbiology and pathogenesis of the disease causing agent and its clinical symptoms and treatments.

Keywords: Anthrax, Bio-terrorism, *Bacillus anthracis*

Introduction

In recent times, the increased threat of terrorism has attracted the attention of society to the risk posed by various microorganisms as biological weapons. Biological warfare agents are more potent than conventional and chemical weapons. During recent past, immense progress in biochemistry, genetic engineering and, biotechnology has simplified the development and production of biological warfare agents. Wide availability and ease of production has increased the demand for biological agents by various developed and developing nations. Among the numerous bioterrorism agents, anthrax may be an effective biological weapon since it is easy to culture, can be aerosolized, and readily forms spores which remain viable for years, infecting soil and other materials long after initial attack (Anonymous, 2004a). Besides this, it can cause wide spread illness and death that can cripple a city or region (Ridel, 2004).

The Center for Disease Control and Prevention (CDC), Atlanta, Georgia has categorized bioterrorism agent into three categories. Organisms in category A (e.g. *Bacillus anthracis*) are high priority because they pose a risk to national security. Category A organisms can be easily disseminated or transmitted from person to person, resulting in high mortality rate. The potential for major public health impact leading to public and social disruption creates the need for public health preparedness. Transmission of *Bacillus anthracis* is well known, and following an intentional release, results in inhalation of anthrax spores (Anonymous, 2004b). Mortality rates in inhalation anthrax patients are very high despite appropriate antibiotic treatment. In 1972, a convention on the "Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction" also

known as BWC, was held. It prohibited offensive bio-weapons research and development and was signed by most countries (Ridel, 2004). However, the former Soviet Union and Iraq, both signatories of the convention, have subsequently acknowledged bio-weapons research and production (Ridel, 2005). Following the World Trade Center attack in September 11, 2001, the "anthrax letter" created widespread psychosocial breakdown and uncertainty. The use of anthrax as a bio-terrorist weapon is likely in coming era. This threat requires the medical community to educate and prepare itself for a response to this possible terrorism.

History

Anthrax is primarily a disease of herbivorous animals, although all mammals (including humans) and some avian species can contract it. The disease has world-wide distribution and is a zoonosis. From the earliest historical records until the development of effective veterinary vaccine and antibiotics, the disease was one of the foremost causes of uncontrolled mortality in herbivores i.e. cattle, sheep, goats, horses and pigs. The major sources of human anthrax infection in its various forms (inhalation, cutaneous and gastro- intestinal) are through direct or indirect contact with infected animals, or occupational exposure to infected or contaminated animal products. Other possible sources are rare and epidemiologically trivial. The bacillus responsible for causing anthrax has a long history of interaction with humans. Described as far back as 1500 BC, it is thought to be the etiologic agent of the fifth Egyptian plague (Anonymous, 2002). It has been suggested in texts of antiquity that the famous plague of Athens (430-427 BC) was an epidemic of inhalation anthrax (Mcsherry and Kilpatrick,1992).The species name is derived from Greek word "anthracites", means coal like, indicative to the typical black eschar seen in the cutaneous form of the disease. There are accurate records of infections in ancient Rome depicted by the Roman Poet Virgil (70-19 BC) in his third "Georgics" which is devoted to animal husbandry and contains a section of veterinary medicine. He detailed an epizootic that occurred in the Noricum district of Rome (ancient name of the Danube River delta and the eastern Alps), where disease affected cattle, horses, sheep as well as dogs and other domestic and wild animals (Virgil, 1956). Over the centuries, it was known as the "Black Bane" and the "malignant pustule" due to its cutaneous manifestations, although it is neither pustulant nor necessarily deadly. Other names include "Rag pickers' disease" Charbon, Milzbrand, "Tanners' disease", Siberian (splenic) fever etc. It was also known as " Wool sorters' disease" because of occupational hazards frequently observed among mill workers exposed to animal fibers contaminated with *B. anthracis* spores (Riedel, 2005; Anonymous, 2002).

In 19th century, anthrax was a major point of interest in biological research. Pierre Rayer and his assistant Casimir – Joseph Davaine in 1850 were the first to observe non motile, filiform bodies in the blood of sheep having died of anthrax. In 1855 Pollender, 1857-58 Brauell, 1859 Fuchs and 1860 Delafond also identified that same agents in anthrax affected animals. Among them Brauell, in 1857-58 first reported the transmission of anthrax from man to sheep. In a series of studies between the years 1863 and 1868, Davaine definitely established the presence of filiform bodies in the blood of animals, which had died of anthrax. He gave the name "bacterides" to these bodies (Merchant and Parker, 1983).

In 1871, Tiegel and in 1877 Louis Pasteur and Joubert, showed that anthrax was caused by the small "bacterides" of Davaine because filtrate from which the organisms had

been removed did not produce disease (Merchant and Parker, 1983). In 1877, Koch postulated that the anthrax bacilli could be transmitted from one host to another and he grew the organism in vitro and induced the disease in healthy animals by inoculating them with materials from these bacterial cultures (Riedel, 2005). He was able to trace the complete life cycle of this bacilli for first time (1876) and also explored that it remain viable for long periods in unfavourable environment (Carter, 1988). Koch proved his postulates by isolating the organism from the infected animal (Merchant and Parker, 1983).

Contributions to the immunity of anthrax were made in 1880 by Chauveau and in the same year by Toussaint. Final and undoubted proof of the value of vaccination, however, resulted from the famous experiments of Pasteur at Pouilly-le-Fort; a small village out-side of Paris, in 1881. He inoculated 25 cattle with his anthrax vaccine, which contained live attenuated organisms. Subsequently, he inoculated the vaccinated as well as other cattle with a virulent strain of anthrax bacilli. All of the vaccinated animals survived, but others died (Merchant and Parker, 1983; Carter, 1988). At this time, both Koch and Pasteur pave the way for further work in medical microbiology.

Anthrax is world wide in its distribution. It is particularly prevalent in countries where no organized control of animal disease exists. India, China, Siberia, Russia, northern Africa, some parts of South America and Mexico has anthrax as a major livestock problem. Germany, France, Italy, Great Britain and the United States keep the disease well under control. Anthrax is enzootic in southern India but is less frequent to absent in the Northern Indian states where the soil is more acid, while in Nepal it is endemic. Hansen believes that anthrax was introduced into the Ohio River Valley during the early days of westward migration, and he cites that Kercheval was the first person to describe an outbreak of the disease in cattle and infection in four farmers in the United States in 1824. An extensive epizootic of anthrax occurred in northeastern Oklahoma and southeastern Kansas in 1957, with a loss of 1627 animals on 741 premises as reported by Van Ness and associates (Merchant and Parker, 1983). As countries become free of anthrax or the annual incidence of outbreaks approaches unity, the numbers of animals, affected in an outbreak increase. This seems to be due to the decreasing veterinary experience in recognizing cases and in dealing appropriately with outbreaks. The mere absence of reported livestock anthrax does not mean that a country is free of the disease. Reporting deficiencies and insufficient examination of unexpected livestock deaths are common throughout the world (Anonymous, 1997 a,b).

Circumstantial evidence indicates that man is moderately resistant to anthrax. Before vaccines and antibiotics became available, and at a time when understanding of industrial hygiene was relatively basic, workers in industrial occupations processing animal products were exposed to significant numbers of anthrax spores on a daily basis. In Britain, 354 cases of Anthrax in such industries were notified during 13-year period 1899-1912 (Anon, 1918). Although the numbers of persons exposed is not known, it must have been many thousands, and the number of cases represented only a very small proportion of the number exposed. With improvements in industrial hygiene practices and restrictions on imported animal products, the number of cases fell in considerable level in latter parts of the 20th Century. However, death rates remained high (>85%) when inhalation anthrax occurred (Riedel, 2005).

Historical analysis of epidemiological data globally reveals the following approximate ratios: a) One human cutaneous anthrax case to ten anthrax livestock carcasses; b) One incident of enteric human anthrax to 30-60 anthrax-infected animals eaten; c) in humans, 100-200 cutaneous cases for each enteric case that occurs. Industrial anthrax incidence data can be inferred from the volume and weight of potentially affected materials handled or imported, taking into account the quality of prevention, such as vaccination of personnel and forced ventilation of the workplace. These relationships are essentially all that can be used for many countries where human anthrax is infrequently, erratically or incompletely reported. In addition, certain countries suppress anthrax reporting at the local or national levels.

Human case rates for anthrax are highest in Africa, the Middle East and central and southern Asia. Where the disease is infrequent or rare in livestock it is rarely seen in humans (Turnbull *et al.*, 2002). In the 1950s, US Army Chemical Corps was developed human anthrax vaccine which was replaced by new and improved vaccine in 1970 with its license. In 1997, the US armed forces mandated vaccination for all reserve and active troops (Riedel, 2005; Morris, 1999). Cutaneous anthrax is said to account for 95% or more of human cases globally. However, serological and epidemiological evidence suggest that undiagnosed low-grade gastrointestinal tract or pulmonary anthrax with recovery can also occur, and may not be infrequent among exposed groups (Brachman *et al.*, 1960; Norman *et al.*, 1960).

An outbreak in a mill in New Hampshire, USA, in 1957 was not associated with any unusual change in occupational exposure, but seems to have been an isolated event within a prolonged period of exposure. The US Department of Defense bases its strategies on an estimate that the LD50 for humans is 8000 to 10,000spores. However, in this relation it was found that workers were found to be inhaling 600 to 1300 anthrax spores over an 8-hour shift without ill effect. *B. anthracis* was recovered from the nose and pharynx of 14 of 101 healthy persons. Furthermore, it is well established that, spores above 5 mm have increasing difficulty in reaching the alveoli of the lung. The likelihood of inhaled spores penetrating far enough to induce inhalation anthrax therefore depends greatly on the size of the particles to which they are attached (Turnbull *et al.*, 2002; Meselson *et al.*, 1994; Dahlgren *et al.*, 1960).

Outbreaks and epidemics do occur in humans, sometimes these are sizeable, such as the epidemic in Zimbabwe which began in 1979, was still smouldering in 1984-85 and had by that time affected many thousands of persons, albeit with a low case fatality rate (Turner, 1980; Davies, 1982).Typically, gastrointestinal anthrax follows the consumption of insufficiently cooked contaminated meat. In 1987, 14 cases of gastrointestinal and oropharyngeal anthrax were reported from northern Thailand (Kunanusont *et al.*, 1989).

Anthrax related biological warfare

If we go through the history of attempts of using diseases in biological warfare, it illustrates the difficulty of differentiating between a naturally occurring epidemic and an alleged or attempted biological warfare attack. This problem has continued into present times. The conception of Koch's postulates and the development of modern microbiology during the 19th century made the isolation and production of stocks of specific pathogen possible. Many countries have worked to develop these agents for biological warfare purpose (Ridel, 2004; Riedel, 2005). So, the use of biological warfare became sophisticated during the nineteenth century.

World War I

Substantial evidence suggests the existence of an ambitious biological warfare program in Germany, England and France during World War-I. This program allegedly featured covert operations. During World War I, reports circulated of attempts by German to ship horses and cattle inoculated with disease producing bacteria, such as *Bacillus anthracis* (anthrax) and *Pseudomonas pseudomalli* (glanders), to the USA and other countries (Riedel, 2005; Hugh-Jones, 1992). Though no hard evidence of using such arms was found, an international diplomatic meeting was held on 17th June, 1925. The "Protocol for the Use in War of Asphyxiating, Poisonous or Other Gases and of Bacteriological Methods of Warfare" (aka Geneva Protocol) was signed by 108 nations. Ultimately, it was meaningless since several countries (including Canada, Belgium, France, Great Britain, Italy, the Netherlands, Japan, Poland and the Soviet Union) began to develop biological weapons soon after its official implementation. The USA remained apart from the Geneva Convention until 1975 (Ridel, 2004).

World War II

During World War II Germany, Canada, United Kingdom, Japan, the Soviet Union, and the USA began an ambitious biological warfare research program. Various allegations and counter charges clouded the events during and after World War II. Japanese biological warfare program was known as "Unit 731" and was conducted in occupied Manchuria near the town of Pingfan from 1932 until the end of World War II. The program was under the direction of Shiro Ishii (1932-1942) and Kitano Misagi (1942-1945) and consisted of more than 150 buildings, 5 satellite camps, and a staff of more than 3000 scientist (Eitzen and Takafuzi, 1997; Christopher *et al.*, 1997). *Bacillus anthracis* was one of the organisms of interest which had been extensively researched and used. The Japanese government accused the Soviet Union of experimentation of biological warfare agents *like B. anthracis*, *Shigella*, *V. cholerae* based on recovery of such agents from Russian spies (Ridel, 2004). German medical researchers infected prisoners with disease producing organisms like *Rickettsia prowazeki*, hepatitis A virus, and malaria. Despite this effort, a German offensive biological warfare program was not completely materialized (Hugh-Jones, 1992).

On the other hand, German officials accused the allies of using biological warfare agents as weapons. It was believed by many that the British were experimenting with at least one organism as biological warfare agent (i.e. *Bacillus anthracis)* under the control of Dr. Paul Fildes in 1940s. Bomb experiments of weaponized spores of *B. anthracis* were conducted on Gruinard Island near the northwest coast of Scotland. This led to a heavy contamination of the island with persistence viable spores. In 1986, the island was finally decontaminated by using formaldehyde and seawater (Christopher et al., 1997; Manchee and Stewart, 1988). Offensive Biological warfare program was begun in USA on 1942 at Camp Detrick (recent name Fort Detrick), Maryland where the organism of interest was *B. anthracis*. Canada also started biological warfare program with small number of workers where *B. anthracis* was also under consideration during World War II.

After World War II

During the Korean War, the Soviet Union, China, and North Korea accused the USA of using anthrax spores against the Chinese and North Koreans, although this has not been proved. The credibility of the USA in this respect remained questionable due to its failure to

ratify Geneva Protocol of 1925, and because of suspicions of collaboration with former Unit 731 scientist for its own offensive biological warfare program (Ridel, 2004; Anonymous, 2002).

Biological Weapon Convention 1972

It was obvious that Geneva Protocol was a toothless measure for control & proliferation of biological weapons. Risks of an unpredictable nature as well as lack of epidemiological control measures for biological weapons became a serious concern internationally during late sixties. In 1969, Great Britain and the Warsaw Pact Nations submitted a proposal for the prohibition of biological weapons. This proposal was strengthen by a report issued by World Health Organization on November, 1969 regarding the possible consequences of the use of biological weapons. The report revealed that anthrax is a dangerous threat because it showed that release of 50Kg aerosolized anthrax agents by aircraft over a population center of 500,000 causes 95,000 deaths and 1, 25,000 severe incapacitations (Ridel, 2004; Riedel, 2005; Anonymous ,1971; Anonymous, 1970). In the way of on-going process, in 1972 "Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction" known as the BWC, was developed. The BWC was signed by 103 nations and implemented officially in April 1972. This measure helped to prohibit development, production, stockpiling, and technological transfer of biological warfare agents.

Post BWC Scenario

Despite BWC agreement, some signatories continued their offensive bio-warfare program. In April, 1972 a large epidemic of inhalation anthrax occurred among citizens of Sverdlovsk (now Ekaterinburg), Russia. The epidemic was suffered by those who lived and worked near a soviet military microbiology facility, known as Compound 19. Many livestock also died of anthrax in same area within 50 Km from that Compound in Sverdlovsk. European and US intelligence suspected that this epidemic might be attributed to an accidental release of anthrax spore from Compound 19 where biological warfare research was conducted. In February 1980, the well circulated German daily "Bild Zeitung" revealed a story about the accident in Soviet military settlement in Sverdlovsk in which an anthrax cloud had resulted. Soviet officials attributed the human cases of anthrax from the ingestion of contaminated meat.

The epidemic of Sverdlovsk, which claimed thousands of lives, required independent scientific investigation. In 1986, Matthew Meselson (Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts) conducted several unsuccessful trials, which ultimately led to an invitation to come to Moscow for discussion with 4 Soviet physicians engaged in Sverdlovsk outbreak. The outcome of this meeting was that epidemiological and pathoanatomical data was needed for further investigation. The Soviet Union maintained that this outbreak was due to consumption of anthrax contaminated meat from the black market.

After collapse of the Soviet Union in 1992, the Russian President Boris Yeltsin admitted that the compound 19 was a part of offensive Biological warfare program and the epidemic was caused by an accidental release of anthrax spores. Returning to Russia, Meselson and his team joined in the investigation and reviewed demographic, ecologic, atmospheric as well as private pathologist's data. This led them to the conclusion that the 42 cases of fatal anthrax

bacteremia and toxemia were typical of inhalation anthrax as seen in experimentally infected nonhuman primates. The data indicated that the outbreak resulted from an aerosol that originated from Compound 19. After this incident, the research was continued at a remote military facility in the isolated city of Stepnogorsk in Kazakhstan, for producing more virulent anthrax strain (Ridel, 2004; Riedel, 2005; Messelson *et al.*, 1994; Caidle, 1997).

In August 1991, during the process of UN inspection, representatives from the Iraqi government announced to the UN Special Commission Team that Iraq had conducted research on offensive biological weapons including *B. anthracis*. Later, a bioterrorism attack in Japan using anthrax and botulinum toxin was also conducted unsuccessfully by Aum Shinrikyo before March 1995 (Ridel, 2004; Messelson *et al.*, 1994).

2001 Scenario

Demonstration of "anthrax letters" associated with intentional release of the anthrax organism following World Trade Center attack in September 2001, was first confirmed in a journalist in Florida on October 2001. Later some cases of cutaneous and inhalation anthrax was seen in postal workers who handled those types of mail. Analysis of these cases suggested that this bioterrorism was conducted by using Ames strain of *B. anthracis* (Ridel, 2004; Riedel, 2005).

In November 2, 2001 a sample from an envelope containing suspicious white powder received by the office of Deputy Chief Minister Chhagan Bhujbal has tested positive for the presence of anthrax spores, making this the first confirmed case of "anthrax mail" in India. This sample was confirmed by Molecular Diagnostics Pvt. Ltd. in Thane (Anonymous, 2001a).

Microbiology

The genus *Bacillus* is comprised of about 70 species with diverse characteristics, including: *B. anthracis, B. cereus, B. licheniformis, B. mycoides, and B. thuringiensis*. Members of the *Bacillus cereus* group (B. *anthracis, B. cereus and B. thuringeinsis)* are really pathovars of a single species (Riedel, 2005). Most species are saprophytes with no pathogenic potential. However, they often contaminate clinical specimens and laboratory media. *B. anthracis* is the most important pathogen in the group.

Most *Bacillus* species are large, Gram-positive, endospore forming rods up to 4 to 10 µm by 1-1.5 µm in size. They are aerobic or facultative anaerobic. With the exception of *B. anthracis* and *B. mycoides* they are motile. In blood smears of tissue or lesion fluid from diagnostic specimens, these chains are two to a few cells in length. In suspension made from agar plate cultures, they can appear as endless strings of cells which are responsible for the tackiness of the colonies. Also characteristic is the square-ended i.e. "box car" shaped appearance traditionally associated with *B. anthracis* vegetative cells, although this is not always very clear. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential growth phase in presence of oxygen. Under anaerobic condition the bacilli in infected tissue secretes a polypeptide [poly- (D-glutamic acid)] capsule, but this character is lost when the bacterium is grown aerobically in vitro. The capsule can be induced by incubating in defibrinated horse blood for at least 5 hrs, or by culturing the isolate on

nutrient agar containing 0.7% sodium bicarbonate with incubation at 37°C in presence of 5% to 10% carbon dioxide. This capsule can be identified under microscope by the staining with polychrome methylene blue (McFadyean's reaction), where capsule stains pink and bacillus cells stains dark blue or may be highlighted with negative staining (Riedel, 2005).

Bacillus anthracis grows readily on different types of media at 37°C. A slightly alkaline medium, pH 7.5 to 7.8 is most conclusive to good growth. *B. anthracis* colonies are up to 2 mm to 5 mm in diameter, flat, dry, grayish and with a "ground glass" appearance after incubation for 48 hours. At low magnification, curled outgrowths from the edge of the colony impart a characteristic, "medusa head" appearance. In gelatin stab culture, filaments of growth radiate from the line of puncture and give the appearance of an inverted fir tree. It is generally non-haemolytic on sheep blood agar media. It also shows weak and slow lecithinase activity on egg yolk agar. Biochemically it is non-lactose fermenting, indole and H_2S are not produced, nitrates are reduced to nitrites, and ammonia is produced. The organism is Methyl Red positive and Voges-Proskauer variable. Its diagnostic feature is that it shows Mc Fadyean reactions, susceptibility to diagnostic gamma phage and penicillin and its colony characteristics (Merchant and Parker, 1983;Anonymous, 2001b).

Bacilli will form spores in the environment outer from host body. Under favorable conditions, anthrax spores germinate and rapidly multiply into vegetative form. Little information exists regarding lifestyle of this pathogen outside of the host. Recently it was found that spores of *B.anthracis* have the capacity to germinate in the rhizosphere of grass plants and to establish populations of vegetative cells that could support horizontal gene transfer in the soil and helps in the evolution of this species under *Bacillus cereus* group as a saprophytic organism outside the host (Saite and Kochler, 2006).

Multiple locus enzyme electrophoresis (MEE) and multiple locus sequence typing (MLST) have shown the lack of genetic diversity of *B.anthracis*. It is one of the most molecularly monomorphic bacteria known. Amplified fragment length polymorphism (AFLP) analysis helps to detect difference between *B. anthracis* isolates and to examine phylogenetic relationship between *B. anthracis* and its close relatives. Multiple locus variable number tandem repeat analysis (MLVA) which, unlike AFLP designed to subtype *B. anthracis* specifically and can not be used to address phylogenetic relationship between *Bacillus* species. MLVA determines the copy numbers of variable number tandem repeat (VNTR) in the region of VrrA genes as well as on two plasmids to differentiate into 89 distinct genotypes of these bacilli. Besides this, single nucleotide polymorphism (SNP) also helps in genome-based analysis of bacilli.

Besides this, full confirmation of virulence can be carried out using the polymerase chain reaction (PCR). Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by resuspension of a loop-full of growth in 25 ml sterile deionized (or distilled) water and heating to 95° C for 10 minutes. Following cooling to approximately 4° C, and brief centrifugation, the supernatant can be used for the PCR reaction (Table-1 for primers information). PCR can be carried out in 50 ml volumes using the above primers, 200mM each of dATP, dCTP, dTTP and dGTP, 1.5mM MgCl and 2.5 units of Amplitaq polymerase, all in NH_4 buffer, followed by addition of 5 ml of template DNA.

Alternatively,"Ready-To-Go™" beads are available from Pharmacia Biotech (Uppsala, Sweden, product number 27-9555-01). These are premixed, predispensed, dried beads, stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 ml PCR reactions. The template can be added in a 2.5 ml volume. For strains of *B. anthracis* lacking both pX01 and pX02, the primers specific to the S-layer can be included to confirm the presence of *B. anthracis* chromosomal DNA (Table-1). It is probably advisable to run the S-layer primers alone rather than in multiplexed with PA and Capsule primers. The following PCR cycle can be used: 1 x 95° C for 5 minutes; 30 x (95° C for 0.5 minutes followed by 55° C for 0.5 minute followed by 72° C for 0.5 minute); 1 x 72° C for 5 minutes; cool to 4° C (Turnbull *et al.*, 2002).

Table 1. Suitable primers for confirming the presence of pX01 and pX02 plasmids

Target	Primer ID	Sequence 5´-3´	Product size	Conc.
Protective Antigen (PA)	PA5 3048-3029 PA8 2452-2471	TCCTAACACTAACGAAGTCG GAGGTAGAAGGATATACGGT	596bp	1mM
Capsule	1234 1411-1430 1301 2257-2238	CTGAGCCATTAATCGATATG TCCCACTTACGTAATCTGAG	846bp	0.2mM
S-layer	Upper 391-413 Lower 1029-1008	CGCGTTTCTATGGCATCTCTTCT TTCTGAAGCTGGCGTTACAAAT	639bp	0.2mM

Pathogenesis

The virulence of *B. anthracis* derives from the presence of a capsule and the ability to produce a complex toxin. Both virulence factors are encoded by plasmids and are required for disease production. The expression of virulence factors is regulated by host temperature and carbon dioxide concentration. The capsule is composed of high molecular weight poly peptide (poly-D-glutamic acid) and is encoded by the pXO2 plasmid. This small plasmid is 95.3 kilo base pairs and encodes the three genes *capB*, *capC*, *capA*. The capsule inhibits phagocytosis of the vegetative form of *B. anthracis*.

Another plasmid pXO1 is of 184.5 kb pairs and encodes toxin complex, which consists of three synergistically acting proteins i.e., Protective Antigen (PA,83 KDa), Lethal Factor (LF, 87 KDa) and Edema Factor (EF, 89 KDa) is produced during the log phase of growth of *B. anthracis* (Riedel, 2005; Turnbull *et al.*, 2002). Recent study provides evidence that pXO2 is necessary for the maximal expression of pXO1(Lamonica *et al.*, 2005). Individually each factor lacks toxic activity in experimental animals, although protective antigen induces antibodies which confer partial immunity. LF in combination with PA forms lethal toxin and EF in combination with PA for oedematoxin. Both these toxins are now regarded as responsible for characteristic signs and symptoms of anthrax.

According to the currently accepted model, Protective Antigen binds to receptors on the host cells and is activated by a host protease which cleaves off a 20 KDa piece, thereby exposing a secondary receptor site for which LF and EF compete to bind. The PA+LF or PA+EF are then internalized and the LF and EF are released into the host-cell cytosol. Edema

Factor or EF is a Calmodulin-dependent adenylate cyclase which by catalyzing the abnormal production of cyclic-AMP (cAMP) produces them altered water and ion movements that lead to the characteristic oedema of anthrax. High intracellular cAMP concentrations are cytostatic but not lethal to host cells. EF is known to impair neutrophil function and oxidative process and its role in anthrax infection may be to prevent activation of the inflammatory process.

Lethal Factor or LF appears to a Calcium and Zinc- dependent metalloenzyme endopeptidase. It has recently been shown that it cleaves the amino terminus of two mitogen-activated protein kinases and thereby disrupts a pathway in the eukaryotic cell concerned with regulating the activity of other molecules by attaching phosphate groups to them. This signaling pathway is known to be involved in cell growth and maturation, but the manner in which its disruption leads to the known effects of LF has yet to be elucidated. On the basis of mouse and tissue culture models, macrophages are major target of lethal toxin which is cytolytic in these. The initial response of sensitive macrophages to lethal toxin which is the synthesis of high level of Tumor Necrosis Factor i.e., TNF alpha and Interleukin-1 beta cytokines and it seems probable that death in anthrax infections results from a septic shock type mechanism resulting from the release of these cytokines.

The endothelial cell linings of the capillary network may also be susceptible to lethal toxin and the resulting histologically visible necrosis of lymphatic elements and blood vessel walls is presumably responsible for systemic release of the bacilli and for the characteristic terminal hemorrhage from the nose, mouth and anus of the victim (Turnbull *et al.*, 2002).

Infection with anthrax occurs after introduction of spores through a break in skin in case of cutaneous anthrax or entry through the mucosa in case of gastrointestinal anthrax. After spores are ingested by macrophages at the port of entry, germination of the vegetative form occurs and the bacilli rapidly multiply. Rapid extra cellular multiplication is accompanied by the production of the lethal toxin and oedema factor.

In inhalation anthrax, spores (1-2 µm in diameter) are inhaled and deposited in the alveolar spaces. From there, they are transported to local lymphatic and the mediastinal lymph nodes, where they germinate and cause hemorrhagic lymphadenitis. Vegetative bacilli then further spread via the blood stream and lymphatics, causing septicemia. The large amount of toxin produced by the bacilli, together with the host response i.e., release of TNF-alpha and Interleukin-1 are responsible for the rapid decline and the overt symptoms of the host organism. Recently, several newly identified putative virulence factors were observed, these include enolase, high affinity zinc uptake transporter, the peroxide stress related alkyl hydogen peroxide reductase, isocitrate lyase, and the cell surface protein A (Riedel, 2005; Lamonica *et al.*, 2005).

Clinical symptoms

Anthrax in human is classically divided in two ways. The first type of classification reflects how the occupation of the individual led to their exposure to anthrax. The non-industrial type occurrs in farmers, butchers, knackers, veterinarians and so on. The other type is industrial anthrax, occurring in those employed in bones, hides, wool and other animal products. Industrial anthrax distinguishes between cutaneous anthrax acquired through a skin lesion, or insect bite,

gastrointestinal tract anthrax contracted from ingestion of contaminated food, primarily meat from an animal that died of disease, or conceivably from ingestion of contaminated water and pulmonary (inhalation) anthrax from breathing in air borne anthrax spores.

Cutaneous anthrax is said to account for 95% or more of human cases globally. In cutaneous cases, incubation period ranges as little as 9 hours to 2 weeks, mostly 2 to 7 days. *B. anthracis* (usually as spores) entered through skin lesion like cut, abrasion, insect bite etc. A small pimple or papule appears; gradually a ring of vesicles develops around it. Marked oedema starts to develop. Painful lymphadenitis may occur in the regional lymph nodes. The original papule ulcerates to form the characteristic eschar. Oedema extends some distance from the lesion. Clinical symptoms may be more serve if the lesion is located in the face, neck or chest. In these more serve forms, clinical findings are high fever, toxemia, regional painful adenomegaly and extensive oedema, shock and death may ensue. Generally, eschar resolves within six weeks. In untreated cutaneous anthrax, about 20% of patients develop septicemia and die. However, with the use of appropriate antibiotics, the mortality rate is < 1% (Lew, 1995). In this context, seven confirmed and four suspected cases of cutaneous anthrax were identified during the 2001 out break. Skin trauma was not associated with these cases of cutaneous anthrax. Exposure to contaminated mail was the apparent source of infection in all patients.

There are two clinical forms of gastrointestinal anthrax which may present following ingestion of *B. anthracis* in contaminated food or drink. Intestinal forms include symptoms like nausea, vomiting, fever, abdominal pain, haematemesis, bloody diarrhea and massive ascites. Unless treatment commences early enough, toxemia and shock develop, followed by death. There is evidence that mild, undiagnosed cases with recovery can occur (Brachman *et al.*, 1960). In this form ulcers and necrosis usually form in the wall of the terminal ileum; sometimes caecum, colon, stomach and duodenum can also be involved (Riedel, 2005). In oropharyngeal form, the main clinical features are sore throat, dysphasia, fever, regional lymphadenopathy in the neck and toxemia. Even with treatment, the mortality is about 50% (Doganay *et al.*, 1986). The suspicion of anthrax depends largely on awareness and alertness on the part of the physician as to the patient's history and to the likelihood that he/she had consumed contaminated food or drink.

In case of pulmonary form of anthrax symptoms prior to the onset of the final hyperacute phase are non-specific and suspicion of anthrax depends on the knowledge of the patient's history. Early symptoms are non-specific and "flu-like" begin insidiously with mild fever, fatigue lasting one to several days (Plotkin *et al.*, 1960). Headache, muscle aches, chills, fever, drenching sweats, minimally productive cough, nausea or vomiting, mild chest pain were symptoms recorded in diseased patient's chest radiography at initial examination showed mediastinal widening, paratracheal fullness, hilar fullness, pleural effusions or infiltrates or both; chest computed tomography scan is helpful in detecting hemorrhagic mediastinal lymph nodes and oedema, peribronchial thickening, hyper dense mediastinal and hilar adenopathy findings seen in inhalation anthrax. This mild initial phase was followed by the sudden development of dyspnoea, cyanosis, disorientation with coma and death. Death occurred within 24 hours onset of the hyper acute phase. In 2001 outbreak of bioterrorism related anthrax 11

patients were identified for inhalation anthrax are believed to have been exposed to mail containing or contaminated with *B. anthracis* spores.

Meningitis due to anthrax is a serious clinical development which may follow any of the other three forms of anthrax. The case fatality rate is almost 100%, the clinical signs of meningitis with intense inflammation of meninges, markedly elevated CSF pressure and the appearance of blood in the CSF (the meningitis of anthrax is a hemorrhagic meningitis) are followed rapidly by loss of consciousness and death (Turnbull *et al.*, 2002).

Sepsis develops after the lymphohaematogenous spread of *B. anthracis* from a primary lesion (cutaneous, gastrointestinal or pulmonary). Clinical features are high fever, toxemia and shock, with death following in a short time (Turnbull *et al.*, 2002). Where anthrax has not been suspected prior to post mortem, characteristic signs are dark haemolysed unclotting blood, enlarged hemorrhagic spleen, petechial hemorrhages throughout the organs and a dark edematous intestinal treat, ulcerated or with areas of necrosis (Turnbull *et al.*, 2002). Histopathological findings from patients with disseminated anthrax showed hemorrhage, oedema, necrosis, fibrin deposition and a variable degree of inflammatory cell infiltrate, predominantly consisting of neutrophils (Abramova *et al.*, 1993).

Treatment and Prophylaxis

Prompt and timely antibiotic therapy usually results in dramatic recovery of the individual or animal infected with anthrax. Almost all isolates of *B. anthracis* can be expected to be highly sensitive to penicillin and being cheap and readily available in most parts of the world, this remains the basis of treatment schedules, particularly in animals and in humans in developing countries. In mild uncomplicated cases of cutaneous anthrax, Penicillin V, 500 mg orally every 6 hours for 5 to 7 days is adequate, but the treatment usually recommended is 3 to 7 days of intramuscular procaine penicillin, 600 mg (1 million units), every 12-24 hours or intramuscular benzyl penicillin (penicillin G), 250,000 units at 6 hours intervals. Cutaneous lesions usually become sterile within the first 24 hours of such regimens, and early treatment will limit the size of lesion.

In severely affected patients or when pulmonary or gastrointestinal anthrax is suspected, the initial treatment is penicillin G, 1200 mg (2 million units per day, by infusion or by slow intravenous injection (< 300 mg / min) every 4-6 hours until the patient's temperature returns to normal. At this point treatment should continue in the form of intramuscular procaine penicillin. Streptomycin, 1-2 gm per day intramuscularly, may act synergistically with penicillin. In the event of allergy to penicillin, several antibiotics are effective alternatively such as tetracycline, chloramphenicol, gentamicin and erythromycin. Trimethoprim is not effective.

In case of anthrax meningoencephalitis suggest that penicillin (2 million units of crystalline penicillin intravenously every 2 hours initially) remains the antibiotic of choice because it diffuses in the cerebrospinal fluid through highly inflamed meninges. Chloramphenicol (1 gm intravenously every 4 hours) is a suitable alternative for hypersensitive patients. Essential supportive therapy includes the early institution of oedema measures, such as 100 ml of 20% manitol intravenously every 8 hours and hydrocortisone, 100 mg every 6 hours (Turnbull *et al.*, 2002).

All the *B. anthracis* isolates associated with 2001 bioterrorism related anthrax outbreak were sensitive to the quinolones, rifampin, tetracycline, vancomycin, imipenem, meropenem, chloramphenicol, clindamycin and the aminoglycosides. The isolates have intermediate-range susceptibility to the macrolides but are resistant to extended-spectrum cephalosporins, including third generation agents like ceftriaxone, and to trimethoprim- sulfamethoxazole 9 (Anonymous, 2001c).

The decision regarding the use of penicillins or amoxicillin, the drugs historically used for treatment and prophylaxis of anthrax, is complicated against the Ames strains causing these recent infection (Riedel, 2005; David *et al.*, 2002). Genomic sequence data show two beta-lactamases: a potential penicillinase (class A) and a cephalosporinase (class B). Concern about the use of penicillin arises because an inducible penicillinase could be activated in the face of treatment with beta-lactams, particularly if the number of organisms present is high, as appears typical with inhalation disease. Concerns have also been raised about the poor penetration of beta-lactams in macrophages, the site where *B. anthracis* spores germinates (David *et al.*, 2002).

Ciprofloxacin and doxycycline may be recommended as a good first line agent. Thus recommendation for initial treatment of inhalation anthrax is a multidrug regimen of ciprofloxacin or doxycycline along with one or more agents to which the organisms is typically sensitive, this treatment also allows empiric coverage for other pathogens. Clindamycin has been suggested to have antitoxin properties. Steroids have been used to control oedema of cutaneous disease and have been suggested for the treatment of meningitis or substantial mediastinal oedema.

During the recent bioterrorist attacks, interim Center for Disease Control and Prevention (CDC), Atlanta recommendation for anthrax prophylaxis include ciprofloxacin or doxicyclin, this are also approved by the Food and Drug Administration (FDA), for prophylaxis of inhalation anthrax infection. The optimal duration of prophylaxis is uncertain, however, 60 days was recommended, primarily on the basis of animal studies of anthrax deaths and spore clearance after exposure. In one human case during the Sverdlovsk outbreak in former Soviet Union on 1979, anthrax developed 43 days after spores were release into the atmosphere (Friendlander *et al.*, 1993). It has been postulated that germination of dormant spores may occur as late as 60 days after infection (Riedel, 2005). There options are now offered for prophylaxis: i) 60 days of antibiotic prophylaxis; ii) 100 days of antibiotic prophylaxis and iii) 100 days of antibiotic prophylaxis, plus anthrax vaccine as investigational post-exposure treatment [3 doses over a 4 week period] (Anonymous, 2001d).

Normally, vaccination seems the most effective form of mass protection. Pasteur produced the first anthrax animal vaccine in 1881. Four decades of research have shown that protection against anthrax in the susceptible host is dependent almost entirely on that host is immune response to a single antigen – the protective antigen (PA) component of the anthrax toxin, a well defined protein of molecular weight 83 KDa. The effectiveness of both animal and human vaccines is dependent on the induction of anti- PA antibodies. However, the immune response is complex and involves cellular immunity in some as yet unidentified manner (Turnbull *et al.*, 2002).

Most anthrax vaccines in use today utilize the toxigenic, non-capsulating (pXO1+ / pXO2-) *B. anthracis* strain 34F2 isolated in 1937 by Sterne. Earlier Pasteur – type vaccines are still in Italy. In Central and Eastern Europe, an equivalent pXO2 – derivative, "Strain 55", is the active ingredient of the current livestock vaccine. Another Strain "1190R- Stamatin" is used for vaccine production in Romania (Turnbull *et al.*, 2002). A vaccine for use in humans was licensed in 1970 by the Food and Drug Administration. This vaccine was based on cultures in a synthetic medium, which was initially termed "528 medium" (Riedel, 2005). In China and in the countries of the former USSR, live spore vaccines are prepared for human use from Strain A16R and Strain STI (Sanitary Technical Institute) respectively (Turnbull *et al.*, 2002). In most other countries, live spore vaccine are not licensed for use in humans. In the United Kingdom and USA, non-living human vaccines developed in the 1950s and 1960s are available (Turnbull *et al.*, 2002). The UK vaccine is an alum-precipitated cell- free culture filtrate of Strain 34F2 while the US vaccine is an aluminum hydroxide – adsorbed cell free culture filtrate of a non-capsulating, non-proteolytic derivative of bovine isolate V770.

Considerable effort has been made in recent years to develop newer and safer anthrax vaccine free from any dangerous side effects in any species. Future vaccines are likely to take two forms: i) vaccines that contain recombinant protective antigen, ii) genetically manipulated vaccines that either contain deletions of genes for PA which in fact helps in deletion of undesired toxin factors or recombinant constructs of PA gene. In this context a new type of plant based vaccine was prepared very recently where PA gene was inserted into chloroplast of the cell of tobacco plants and each plant produce 150 milligrams of PA antigen, which adds up to 360 million doses worth of PA from one acre of tobacco plant and this vaccine is much cleaner than recently used vaccines (44). The aims for prospective new vaccine are that they should be cheap, environmentally acceptable, orally administrable, safe and protective after a single dose (Turnbull *et al.*, 2002).

Conclusion

Though anthrax is a common and naturally occurring disease in domestic animals, but it is gradually coming up on the surface as a devastating biological weapon. Research and development of anthrax as a biological weapon during the first half of the 20th century focused on easy dissemination of the bio-weapon and the development of multidrug resistant strains (Riedel, 2005). But this biological weapon would not only cause sickness and death but also aim to create fear, panic and paralyzing uncertainty. Its goal is disruption of social and economic activity, the breakdown of government authority and the impairment of military responses. Besides this, anthrax causes residual contamination of the ground for a long period along with great devastation in the civilized population as estimated by WHO in 1970 (Ridel, 2004).

Recently demonstrated by the "anthrax letter" in the aftermath of the World Trade Centre attack in September 2001, the occurrence of only a small number of infections can cause an enormous psychosocial breakdown. More importantly, these attacks fueled fears that future attacks might be more extensive.

Despite improvements in treatment and prophylaxis, anthrax is considered a fatal infection. Biological warfare attacks with anthrax agents are now a serious possibility. Primary prevention depends on creating a strong global norm that stops development of such weapons.

Secondary prevention depends on early detection and its prompt treatment. As BWC is preparing to assist nations that have been targets of biological weapons, the medical community must be ready to face the challenge of biological warfare because nobody knows what will happen next.

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