Part A
Pathogens

BSL3 and BSL4 Agents: Epidemiology, Microbiology, and Practical Guidelines, First Edition.
Edited by Mandy C. Elschner, Sally J. Cutler, Manfred Weidmann, and Patrick Butaye.

Part I
Bacteria

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1
Bacillus anthracis: Anthrax

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1.1
Introduction

Anthrax is an acute infection, caused by the bacterium *Bacillus anthracis*. This zoonosis can be transmitted from grass-eating animals or their products to humans. However, it should be noted also that *B. anthracis* has all the characteristics of an environmentally adapted bacterium. Normally, anthrax occurs in 95% of all human reported cases as cutaneous anthrax, caused by bacteria penetrating the skin through wounds or micro fissures, but the bacterium can also manifest in the mouth and the intestinal tract. Infection of the lungs after inhalation of the spores is very rarely observed [1, 2].

B. anthracis is listed as a category A bioterrorism agent by the Centers for Disease Control and Prevention [3]. Besides the knowledge that *B. anthracis* has a history as a biological weapon in different national military programs, the general public became aware of this pathogen in 2001 when specially processed spore-filled letters were sent across the United States of America, killed five persons and infected up to 26. Since that time ''white powder'' became a synonym for the bioterroristic threat all over the world. In addition to this special aspect, anthrax is still an endemic disease in many countries and sporadically occurs all over the world [2]. Today, imported wool or hides from ruminants (e.g., goats, sheep, cows) could be contaminated with spores and occasionally lead to infections in industrial (''wool sorting factory'') or private settings (Bongo drums) in countries where the disease is absent or infrequent [4, 5].

1.2
Characteristics of the Agent

B. anthracis is a nonmotile, Gram-positive, spore-forming aerobic rod, arranged in long chains within the tissue. Sporulation occurs in the presence of free oxygen and endospores develop in central positions that are considered as infectious particles.

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They are extremely resistant to harsh environmental conditions, such as heat, dehydration, pH, desiccation, chemicals, irradiation, or ice. In this dormant state, they can survive up to decades or centuries in the environment without loss of virulence [1, 2]. Robert Koch discovered this agent and its sporulated form in 1877 and demonstrated for the first time his so-called postulates that establish a causal relationship between a pathogen and a disease [6].

1.3 Diagnosis

1.3.1 Phenotypical Identification

B. anthracis belongs to the *B. cereus* group, which comprises six closely related members: *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihenstephanensis*. These species are phylogenetically and phenotypically closely related and their phenotypical identification can be complex, especially because some isolates may display unusual biochemical and/or genetic properties that complicate their distinction. Furthermore, several of these species and also yet-undefined species of this group are normal contaminants in environmental samples such as soil or dust.

Phenotypical identification is the classical method of identification in routine laboratories. Some simple characteristics have been useful for distinguishing between different *Bacillus* species, but they have limitations particularly in distinguishing between *B. cereus* and *B. anthracis* [7]. The following sections focus on the different methods and highlight their advantages and disadvantages.

1.3.2 Growth Characteristics

B. anthracis is a Gram-positive aerobic spore-forming rod which grows well on all types of blood-supplemented agars. After cultivation at 37 °C overnight, *B. anthracis* shows the following morphological characteristics [2]:

- No hemolysis on blood agars;
- Spike-forming (see Figure 1.1);
- ''Sticky'' colonies (see Figure 1.2);
- Medusa head;
- While growing on Cereus-Ident agar (Heipha, Germany), it forms colonies that are silver-gray, silky-shiny, and polycyclic without any pigmentation [8]. As an alternative, trimethoprim-sulfamethoxazole-polymyxin-blood agar (TSPB agar) can be used, containing several antibiotics and resulting in a high selectivity against Gram-negative bacteria.

Figure 1.1 Spike-like projections at the colony edge from *Bacillus anthracis* (in addition no hemolysis on Columbia agar; Bundeswehr Institute of Microbiology, Munich, Germany).

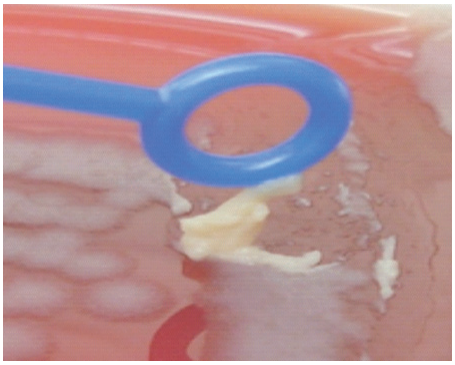

Figure 1.2 "Sticky colony" phenomenon of *B. anthracis* after manipulation with a loop (J. Frey, Bern, Switzerland).

Even if only some of these features are observed, the results should be considered as suspicious for *B. anthracis* and further analysis for differentiation should be mandatory.

1.3.3 Antibiotic Resistance

The so-called "string of pearls"/"Perlschnur" test is based on the fact that most of the strains isolated in nature are sensitive to penicillin (in contrast to *B. cereus* isolates) and show a special stress form of the bacteria when it is faced with sublethal doses of the antibiotic. This very simple test is still used today to get a first very easy confirmation of suspicious colonies.

The determination of the pattern of antibiotic resistance is of utmost interest, primarily for the assessment of a potential release of bacteria with a bioterroristic

background [9], with genetically modified strains, and to detect rarely occurring natural resistances. To establish the antibiotic resistance profiles of strains, the minimum inhibition concentrations (MICs) have to be tested. They are fixed by the Clinical and Laboratory Standards Institute (CLSI). The CLSI publishes guidelines containing protocols and breakpoint values to evaluate antibiotic resistances profiles [10, 11].

1.3.4 Phage Testing and Biochemistry

Phage testing and biochemical tests are methods that are still in use today in veterinary diagnostic laboratories. Biochemical tests do not lead to a clear and valuable result for differentiating *B. anthracis* from *B. cereus*, while phage testing with bacteriophage gamma is more specific. The feature of sensitivity of *B. anthracis* to gamma phage is still used for identification although exceptions were described in this case, too. In former times veterinary laboratories used a mouse inoculation test for confirmation. When all phenotypic characteristics were present and the phage test was positive, and finally the mouse died within one day after intraperitonal inoculation, and bacillus-like bacteria were seen on colored blood samples, the isolated bacterium was seen as a virulent strain of *B. anthracis*.

1.3.5 Antigen Detection

Newer methods have been developed for the identification of *B. anthracis* from the presence of specific surface proteins. Such systems are encapsulated in lateral flow assays, commercialized for example by the companies Tetracore or Cleartest. Even for these assays, some isolates of *B. cereus* that result in a weak positive signal have been described. Based on their low sensitivity, it is recommended to use this kind of experiment only as a confirmation test of suspect colonies rather than for the detection of *B. anthracis* in environmental samples, since the concentration of bacteria may not be adequate in such samples. The advantage of such a test is the ease of specimen handling. Suspicious material is rubbed into a buffer and the suspension is dropped onto the cartridge. After 15 min of incubation, the results can be evaluated like a pregnancy test. If the test is used for checking questionable colonies (especially when they show no hemolysis on blood agar), it gives very specific results in the identification of *B. anthracis*.

1.3.6 Molecular Identification

1.3.6.1 Virulence Plasmid pXO1

The virulence plasmid pXO1 is said to be ''*anthracis*-specific.'' The genes coding for ''lethal factor'' (LF; *lef*), ''edema factor'' (EF; *cya*), and ''protective antigen'' (PA; *pagA*) are normally present on this plasmid and are major virulence factors

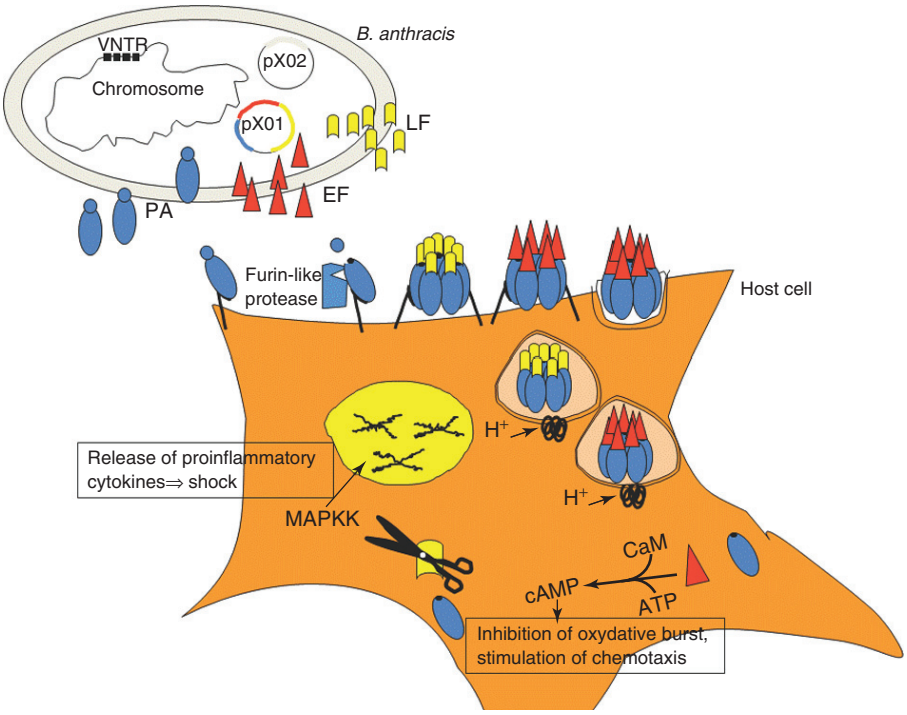

Figure 1.3 Molecular mechanism of virulence of *B. anthracis*. The bacterium *B. anthracis* (upper left), which is protected against phagocytosis by the capsule, a poly-D-glutamic acid polymer, (gray) encoded on plasmid pXO2 secretes an adhesion peptide or protective antigen (PA), the edema factor (EF), and the lethal factor (LF) whose genes are encoded on plasmid pXO1. PA binds to specific cell receptors of the host cell (lower right) where it is processed by a furin-like protease from the host, a process that will lead to multimer formation of PA and association of EF or LF. The PA::EF or PA::LF complexes are taken up by the host cell by endocytosis and are subsequently released in the cell at low pH, when the complexes dissociate and release active EF, a calmodulin-dependent adenylate cyclase, which leads to an inhibition of oxidative burst and an overstimulation of chemotaxis, and actives LF, a zinc-metalloprotease that cleaves mitogen activated protein kinase kinase (MAPKK), which leads to the overproduction and release of pro-inflammatory cytokines and resulting shock. (Courtesy of Elsevier and P. Pilo 2011, see Ref. 30).

of *B. anthracis* (see Figure 1.3). Due to this, most of the commercially available PCR diagnostic kits target sequences of these genes to detect *B. anthracis*. Recently, strains of *B. cereus* could be isolated from patients presenting the clinical symptoms of an "anthrax-like" disease. In these strains at least one of the above-mentioned genes could be observed. Therefore, this plasmid cannot be used as a specific marker for *B. anthracis*, thus complicating the definition and differentiation of species belonging to the *B. cereus* group [7, 12–16]. However, from a clinical point of view, it is not important which species is present or not if these virulence genes are present and the patient's symptoms are similar to those caused by *B. anthracis*.

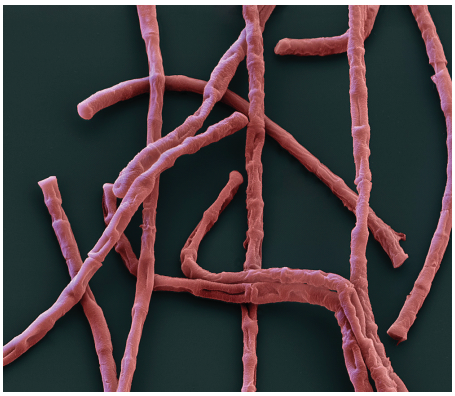

Figure 1.4 Scanning electron microscopic image of *B. anthracis* (6000×) with typical bamboo stick-like appearance of the rods (courtesy of Eye-of-Science, Reutlingen, Germany, 2008).

1.3.6.2 **Virulence Plasmid pXO2**

The virulence plasmid pXO2 contains the genes coding for the capsule-forming enzyme complex (see Figure 1.4). In contrast to *B. cereus*, *B. anthracis* forms a capsule composed of poly-gamma-D-glutamic-acid that is suspected to enable the survival of the bacteria during phagocytosis. The genes *capA*, *capB*, and *capC* are involved in capsule biosynthesis and are normally used as targets for detection by different PCR kits of *B. anthracis*. *B. cereus* does not carry this plasmid or only genetically related versions containing parts or variants [7, 12, 13, 16]. Therefore, PCR detection of both toxin genes on pXO1 and capsule genes on pXO2 is recommended for the identification of *B. anthracis* isolates.

1.3.7 **Chromosome**

A lot of specific targets for the reliable detection and identification of *B. anthracis* have been published. Most of them showed false-positive results during or even after the evaluation of a panel of *B. cereus* strains. The necessity of an unambiguous chromosomal target is due to the presence of so-called "*anthracis*-specific" genes in *B. cereus* and because the *rrs* (16S rRNA) gene sequencing does not provide the information needed. Even the other "classical" targets for species differentiation fail in this specific case and do not provide discriminatory power. Genes encoding the β subunit of the RNA polymerase (*rpoB*) and the subunit A of the DNA-gyrase (*gyrA*) do not display extensive DNA polymorphism between *B. cereus* and *B. anthracis*. The World Health Organization (WHO) recommends the use of the gene *saspB* as a reliable target and has published primers and probes for an assay based on Roche's Lightcycler technology [2]. A Taqman probe-based assay for the detection

of another chromosomal target was published recently [17]. Both assays are up to date without any failure in the identification of *B. anthracis* and differentiation with *B. cereus*.

1.3.8 MLVA, SNR, and SNP Typing

In the case of outbreak investigations but also in forensic examinations of historical or unusual isolates, molecular typing is envisaged and the obtained information can be compared with data retrieved from previously isolated strains [18]. The results of such tests can be used to trace back the origin of the investigated isolates.

In variable number tandem repeats (VNTRs), multi-locus variable analysis (MLVA), or single nucleotide repeat (SNR) analysis, the gene loci comprising repeated motifs bordered by conserved DNA regions are investigated. The combined lengths of the examined loci are likely to differ from strain to strain. The length of the repeated units varies from one base in the case of SNR to several dozens (macro-satellites) in *B. anthracis*. In other organisms, such as *Enterococcus* species, repeat units may harbor up to 300 bp. When the length of the amplified fragment is determined and the amount of the repeated units calculated, the obtained data are comparable to databases similar to paternity testing in humans. Using this strategy, strains can be sourced down to the isolate level [5, 18–21].

Discrimination at cluster or group level is possible using the single nucleotide polymorphism (SNP) typing method. Using this tool, point mutations are investigated and compared to various established populations and, based on the obtained information, they are grouped into different clusters [5].

Such typing techniques can be performed in a reliable way only in specialized laboratories which are experienced in the generation of typing data and the interpretation of them. The use of standardized protocols is highly recommendable in order to correlate data from *B. anthracis* strains isolated worldwide and to trace back their origin [2, 16, 18, 22].

1.3.9 Serological Investigations

Due to the rapid clinical course of systemic anthrax, antibody detection is not a useful diagnostic of acute disease. To control titers after vaccination or to confirm an anthrax case suspicion *a posteriori*, specific antibodies directed against PA could be checked. A commercial enzyme-linked immunosorbent assay (ELISA) for that issue is available on the market (Virion/Serion, Würzburg, Germany). Questionable or very low titer results should be confirmed by blotting techniques [23]. Bead-based assays will be available in the future which could be powerful enough to overcome some problems in sensitivity and specificity [24].

1.4 Pathogenesis

1.4.1 Animals

Mainly ruminants are affected by this disease. The following animal species are categorized in order of susceptibility to the infection: ruminants (bovines, sheep, goat, camel, antelopes), horses, pigs, humans, carnivores (cats, dogs, lions, etc.), birds, amphibian, reptiles, and fish. The latter four in general are little susceptible [25].

In general herbivores get infected by spores from the ground penetrating through micro fissures in the mouth that arise by dryness or by overgrazing on hard and spiny range land. In the lesion, the spores germinate and multiply. After passing the local lymph barriers, sepsis and death can follow within a period of 6 h to 4 days. In general, animals are found suddenly dead, since the animals die within 1–2 h after the clinical symptoms become obvious. Marks of agony may present on the floor (typically signs of leg movements). Less frequent is the acute form, with death within 48 h after the start of clinical symptoms. Typically there is pre-terminal bleeding from the mouth, nose, and anus; and clotting of the blood may be impaired. This blood is highly contagious by germinated anthrax bacilli that convert rapidly to spores under the influence of oxygen. The contaminated skin and the ground around the animals cause the start of a cycle of infection [1, 2].

Birds seem to be not susceptible to the disease and may even be a vector in spreading the bacterium. Especially birds of prey, eating animals killed by an anthrax infection, may excrete the spores over a long distance [26].

1.4.2 Humans

Humans are less susceptible to anthrax than ruminants. The clinical appearance of anthrax in humans depends on the route of infection. In 95% of the cases, *B. anthracis* invades the deep layers of skin through wounds, micro fissures, or even abrasion sites. Through this route, cutaneous anthrax or pustula maligna develops [2]. The predominant locations are the extremities, face, thorax, and abdomen.

Spores in uncooked meat, penetrating the mucosa of the mouth or pharyngeal areas, are the main cause of oro-pharyngeal and intestinal anthrax. In this case, spores invade the lymph-associated barriers and provoke sepsis. This form of anthrax can be observed in families eating the same meal consisting of insufficiently cooked meat. Inhalational or pulmonary anthrax results from the inhalation of a sufficient amount of spores (about 10^4 for a non-immunocompromized host) that are transported to the ''lymphandenous'' tissue where they proliferate [2]. In former times, inhalational anthrax was a severe problem in wool, leather, and bone meal processing factories, where spores were present in high concentrations

in the dust (hence the former name of woolsorter's disease). Today ''biosafety management strategies'' minimize the danger. In the case of respiratory infections, especially if more than one case occurs, a bioterroristic attack has to be ruled out.

Upon entry by the spores into the body, within a few hours the spores germinate and proliferate. Pathology is based on the release of three virulence factors (proteins): the PA that serves as an adhesion subunit for the two main protein toxins, the EF, and the LF that kills the surrounded tissue cells (Figure 1.3) [1, 2].

Immunity against *B. anthracis* can be acquired during a cutaneous anthrax infection but the duration of the protection is unclear. Survivors of systemic anthrax infections show a high antibody response, especially against PA and LF, comparable to a vaccination and will be protected for a long time.

Commercially available vaccines are based on the PA and generate moderate titers. Vaccination might be useful for persons at elevated risk to get in contact with *B. anthracis* and its spores, like laboratory personal, veterinarians, and workers in the above-mentioned industries. However, in most countries, no human anthrax vaccine is licensed. Since 1998, every soldier of the United States Army who is deployed to Iraq or Afghanistan is vaccinated [27]. The vaccination of cattle is still frequent in countries where the disease is enzootic. The strain Sterne is used worldwide as a live vaccine for ruminants. However, one should notice that vaccine strains have to be preserved cautiously because the emergence of non-immunogenic variants can happen as well as mixing with virulent strains from the field [28].

1.5
Clinical and Pathological Findings

One can distinguish the peracute, acute, and subacute forms, or otherwise also the skin form, the intestinal, and the pulmonary form. The incubation period ranges over 1–60 days, depending on the host and amount of bacteria incorporated.

In humans, cutaneous anthrax is the most common form of anthrax and is normally cured by antibiotic therapy without any complications. Typical lesions show a dark reddened papule, formed within 1–3 days with an aperture of only a few millimeters. During the next day, vesicles may appear at the border of this papule, grow, and merge. This cyst cracks within the next day and discharges a hemorrhagic exudate that is highly infectious. Under the skin, an ulcer is formed with a brown, later black dry ground with an aperture of 0.5–3.0 cm (''anthrax'' originates from the Greek word for coal – describing the color of the skin lesions). At the beginning, this ulcer is painless, but later patients were said to feel a localized heat.

The edema leads to ablation of the skin, like after burning. Without any care, such confined existing pustules vanish within 3–6 weeks with little scarring. Therapy is nevertheless mandatory to prevent systemic spread from the initial infection

site. Cutaneous anthrax does not exist in ruminants, and it is rarely seen in carnivores.

1.5.1 Oropharyngeal Anthrax

In humans this form of anthrax appears after the consumption of insufficiently cooked meat from infected animals. The lesion is visible in the stoma pharynx throat. Sometimes circumorale infections are also possible. Similar to the cutaneous form of anthrax, 1–3 days after infection papules appear, growing and transforming into ulcers, accompanied by heavy edemas and a swelling of the regional lymph nodes in soft tissues. Such a glottis or pharynx edema is a life-threatening complication that needs rapid intervention.

This form is also seen in carnivores eating infected or dead animals. In general these animals are not so susceptible to infection, but throat swelling may cause asphyxia. This is also seen in horses, where also death can occur due to excessive intestinal damage [29].

Oropharyngeal anthrax can also exist in a subacute or chronic form in pigs where swelling of the oropharynx causes dyspnea and dysphagia. Complete recovery is possible in these animals.

1.5.2 Abdominal or Intestinal Anthrax

In this case, an infectious dose of spores is ingested and can pass the gastric acid barrier. Germination takes place in the ileum, with a fast infiltration and expansion within the liver and spleen. The symptoms rapidly worsen, accompanied with body pain, vomiting, and bloody diarrhea. A hypovolemic shock with ascites caused by the toxin leads to death within 48 h.

In pigs, it can cause a severe enteritis, with a possibility for recovery. It should be noted that in this animal species intestinal anthrax is seldom accompanied with splenomegaly since septicemia is rare.

1.5.3 Inhalational or Pulmonary Anthrax

Inhalational anthrax is rare and, when a case appears, the possibility of a bioterroristic attack should be ruled out as soon as possible. However, rare deaths have recently been reported after the processing of Bongo drum animal hides. At the beginning, symptoms are fever, cough, pain, and adynamia. These nonspecific disorders lead to acute mediastinitis, with shock and dyspnea. X-rays show an enlargement of the mediastinum and pleural effusion. The change to a systemic infection with a fulminant meningoencephalitis leads in most cases to death.

1.6 Epidemiology

Anthrax is an acute infection in ruminants, especially for cattle, buffalo, sheep, and goats, but also for horses, zebras, antelopes, and elephants [2]. A sporadic appearance can be observed in rabbits from infected fields and in pigs, dogs, and cats fed with infected meat. However, national programs for the control and eradication of anthrax led to the decrease of its natural incidence in animals and in humans worldwide and restricted the disease to animal-related professions. It is nearly absent in northern, middle, and western Europe. Due to stringent measures when cases are met, the discovery of antibiotics, and the development of an effective vaccine, anthrax is no longer considered as one of the most important causes for economical losses in livestock farming. When a case is encountered, the dead animal cannot be moved, and therefore the autopsy and sampling should happen on the spot. After autopsy, all contaminated material is burned on the spot and afterwards a disinfection of the place is performed using burnt lime. Other animals on the premises are checked every 6 h for any increase in body temperature. When there an increase is seen, intravenous injection with penicillin avoids the development of the disease and prevents the further spreading of spores.

Today, anthrax remains a problem, particularly in areas where surveillance systems, especially veterinary controls, are neglected or interrupted due to war activities or natural catastrophes. Also in areas with extended wildlife, control is difficult and has to rely mainly on the decontamination of sites where dead animals were found. Scavengers are problematic since they are little susceptible to the disease but may spread it. In these areas, the disease is still enzootic.

In Eurasia, anthrax was a severe problem in areas of the former Commonwealth of Independent States (CIS) and Turkey in the nineteenth up to the middle of the twentieth century, especially in livestock farming and the wool or leather processing industry. The development of veterinary controls, a ban on burying animal carcasses, the vaccination of animals, and the use of antibiotics led to the disappearance of anthrax. However sporadic cases of *B. anthracis*-infected animals are known from rural regions all over the world.

Due to a lack of precise data, WHO estimates the number of anthrax human cases to be less than 100 000 per year, which mostly present the cutaneous form. The appearance of this disease is mainly related to strong rainfall and manual agricultural activity (gardening). Human to human infections have not been observed [2].

1.7 Conclusion

Anthrax is still present worldwide sporadically or in some areas endemically. It is largely a ruminant disease in ruminants and for this reason its control should primarily start in animals. Strict sanitary measures are needed to handle it. Indeed,

environmental contamination needs to be quite high to be able to infect an animal. Depending on the species, low (ruminants) or high concentrations of spores are necessary to cause the disease. Fortunately there is no direct host to host transmission. Antibiotic treatment is efficient unless the disease has reached the ''point of no return,'' when too many toxins are in the body (this happens mostly when there is inhalation or ingestion of *B. anthracis* in humans). Nevertheless, this rarely occurs under natural conditions. Animal vaccines are available and are useful to control the disease in ruminants in endemic areas. This simplifies the management of outbreaks but the situation can be complicated in some regions because of political, economical, or natural problems. However, in the end, anthrax in wildlife may be difficult to control.

Laboratory diagnosis may sometimes be tricky because of unusual strains of *B. anthracis* and *B. cereus*. As described above, members of the *B. cereus* group are closely related and some characteristics are sometimes not specific. Besides, discussions are ongoing about *B. cereus* strains harboring *B. anthracis* plasmids, especially when both species show no more differences in the clinical outcome between them. Research is needed to understand the ecological and clinical status of these atypical strains.

Recently, a large amount of effort has been invested for the typing and subtyping of strains and isolates of *B. anthracis*. Worldwide data are becoming available to scientists to compare strains and to understand the epidemiology and ecology of *B. anthracis* [2, 16, 19, 22]. Indeed, due to its particular lifestyle, which passes through a sporulated form that can last for decades, this pathogen is highly monomorphic from a DNA sequence point of view.

Nowadays, one concern would come from specially prepared spores for intentional release. Anthrax is known to have been included in bioweapon programs, principally because of the stability and resistance of the spores. This requires trained personnel and validated protocols in order to identify rapidly and efficiently a criminal act. Another concern comes from the import (sometimes illegal) of non-disinfected animal hides and hairs, which can lead to human exposure in individuals living in countries where the disease is not endemic [4, 28]. Hence, anthrax merits medical alertness and awareness even in countries where the disease is not endemic in order to prevent fatal human infections.

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