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Anthrax undervalued zoonosis

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Abstract

Anthrax is a non-contagious disease, known since ancient times but it became a matter of global public interest after the bioterrorist attacks in the U.S.A. during the autumn of 2001. The concern of politicians and civil authorities everywhere towards this emergency necessitated a significant research effort and the prevention of new bioterrorist acts. But anthrax is primarily a disease that affects livestock and wildlife; its distribution is worldwide; and it can represent a danger to humans but especially when it occurs in areas considered to be free and in atypical seasons and climatic conditions. The atypicality of the phenomenon may lead health workers to misdiagnosis and, consequently, an inappropriate management of affected carcasses with a consequent and inevitable increase in the risk of human infection. This paper emphasizes the importance of increasing attention to this zoonosis. The biggest risk is its underestimation.

Key words: Bacillus anthracis, animal, disease, zoonosis
Introduction and historical perspective

Anthrax is an infectious disease with clinical features overlapping the haemorrhagic fevers with hyperacute or acute symptoms and usually with a fatal outcome. The responsible bacterium, *Bacillus anthracis*, was identified only in the late 19th century thanks to studies by Robert Koch (1876).

Pasteur in 1881, with his famous experiment at Fort Poully, showed the world the opportunity to fight anthrax with preventive vaccination, beginning the era of vaccines. However, *B. anthracis* with its high pathogenicity has aroused also less noble interests. From 1932 to 1945 the bacteriologists of the Japanese Army Unit 731 tested it as a biological weapon on Manchurian prisoners (Hudson *et al.*, 2008). Despite the signing of a treaty banning bacteriological arms, an outbreak of human anthrax occurred in Sverdlovsk, USSR, (now Ekaterinburg, Russia) in April 1979. Soviet officials attributed this to the consumption of contaminated meat, but Western governments believed it resulted from inhalation of spores accidentally released from a nearby military research facility. Tissue samples from 11 victims were obtained and analyzed. Results demonstrated that the entire complement of *B. anthracis* toxin and capsular antigen genes required for pathogenicity were present in the tissues from each of these victims. The DNA analysis demonstrated that at least four different strains of *B. anthracis* were present in the tissue samples (Jackson *et al.*, 1998). The world has learnt much about *B. anthracis* following the bioterrorist attacks in the autumn of 2001 in the United States, where five letters containing small quantities of anthrax spores contaminated more than 30000 people, killed 5 people and infected 17 (Jernigan *et al.*, 2002). These events represent a turning point in the history of highly pathogenic agents and their use as a means of aggression against civil populations: that which until then was considered only a remote probability had become a terrible reality and a dangerous example that could be imitated by anyone.

The concern of politicians and civil authorities towards this new emergency necessitated significant preventative programs against new bioterrorist acts and in planning research projects whose aim was to understand the detailed mechanisms of action of the pathogens, and, through therapeutic and preventive means, to minimize the damage of a possible mass attack. We should not forget that anthrax is a disease known since ancient times and it is still present sporadically and endemically in many areas of our planet. The disease is characterized by outbreaks that usually involve a small number of animals, herbivores that are usually kept on pasture, but it can sometimes turn into an epidemic with potentially very serious consequences for humans.

The infectious agent

*B. anthracis*, belongs to the family of Bacillaceae. It is a Gram positive bacterium, a small rod in shape, aerobic, immobile, capsulated, and forms spores. The bacterium is 1-1.5 μm wide by 5-6 μm in length. In tissue or culture smears other pathological bacteria are found singly, as clusters or as short chains, with
rounded ends, while *B. anthracis* cells with square ends are arranged in long chains that gives them a particular look similar to "bamboo canes" (Fig.1). Outside the body and at temperatures between 14°C and 42°C (optimum between 21°C and 37°C) *B. anthracis* will sporulate (Fig.2). The spores are oval, and are released after lysis of the bacterium. Sporulation is completed within 48 hours, but it does not happen in the presence of high concentrations of CO₂, a condition that occurs in infected putrefacting carcasses.

**Culture media features**

*B. anthracis* grows well in ordinary medium under aerobic or microaerophilic conditions, at temperature between 12°C and 44°C, but optimal growth occurs around 37°C and at a pH of 7.0 to 7.4. On nutrient agar it forms white colonies 3-4 mm in diameter with a rough surface, called "glass beads" (Fig.3), and with irregular margins that if observed at a small magnification have a medusa head appearance. In tryptose broth there is a flocculation, which eventually settles on the bottom, leaving clear liquid above. On blood agar it does not cause haemolysis, unlike other bacteria-like anthracoids, which haemolyze in a more or less obvious way. It is normally sensitive to penicillin and various phages. When grown for 2 weeks at a temperature of 42 °C it will lose its virulence in a specific manner (a.k.a. Pasteur vaccine strains). This weakening is due to the loss of the pXO1 plasmid on which the toxic factors are encoded. The colonies of attenuated strains are smaller, smooth, and convex.

**Resistance**

Vegetative forms are not very robust and they are inactivated within 30 minutes at 60°C -65°C (Turnbull, 1998). The great strength of *B. anthracis* is linked to sporogenesis which is nothing else but a form of bacterial resistance to living under unfavorable conditions. The spores are destroyed only after ten minutes at boiling temperature but can withstand 98°C for 30 minutes (personal observation); they are destroyed in 20 minutes in an autoclave set at 121°C. Spores survive well in dried and salted hides, and at low temperatures. The normal fixation techniques do not kill the spores, which can successfully germinate even after many years, so it is necessary to flame slides several times before assuming the spores are dead. The spores are sensitive to 2%-3% formaldehyde solutions at 40°C for 20 minutes, or 0.25% at 60°C for 6 hours, or at 4% after a contact of at least 2 hours. The spores are destroyed by 5% phenol and mercury chloride, and 1% solutions of caustic soda and potash. Spores in soil can persist for many years, but ultraviolet rays will inactive them in a few hours.
The major virulence factors of *Bacillus anthracis*

The pathogenic action of *B. anthracis* is closely linked to toxic factors, resulting from two plasmids:

- **pXO1**, 182 Kb, on which the genes encoding the oedema factor (EF), the lethal factor (LF) and the protective antigen (PA) are located;

- **pXO2**, 96 Kb, on which the genes encoding the biosynthesis of the capsule are located (Uchida I., *et al.* 1985)

The complete chromosomal and plasmid DNA sequences from multiple strains are available from public databases. *B. anthracis* expresses its pathogenic action mainly through the capsule and the production of a toxic complex consisting of three proteins known as protective antigen (PA), lethal factor (LF), oedema factor (EF).

The PA plays a fundamental role in the toxic action of *Bacillus anthracis*. In fact, this protein of about 83 kDa has no direct toxic action but acts as a "transporter" of the other two toxic proteins. PA binds to two cell surface receptors, the tumour endothelium marker 8 (TEM8) and the capillary morphogenesis protein 2 (CMG2), both of which are expressed on many cell types, including cells of the immune system (Collier and Young 2003; Scoobie and Young, 2005). The proteolytic release of the C-terminal domain (20 kDa) of PA results in spontaneous oligomerization of truncated PA (PA63) into heptamers (PA63)_7, which bind EF and LF (Collier and Young, 2003). The (PA)_7-EF and the (PA)_7-LF complexes enter rafts, inducing their endocytosis inside acidic compartments (Abrami *et al.*, 2005). The low pH of the late endosomal lumen triggers a conformational change of the complex, with insertion of part of PA into the membrane and translocation of EF and LF into the cytosol (Abrami *et al.*, 2005). EF is a calmodulin-dependent adenylate cyclase (Leppla, 1982), which creates a gradient of c-AMP with a high concentration in the perinuclear area (Dal Molin *et al.*, 2006), while LF is a metalloprotease which cleaves most isoforms of MAPKKs (MEKs) throughout the cytosol (Vitale *et al.*, 2000); this does not exclude that it may act on other cytosolic proteins, as suggested by a recent report that LF acts on the inflammasome (Boyden and Dietrich, 2006). MEKs are part of a major signalling pathway linking the activation of membrane receptors to transcription of several genes, including those encoding pro-inflammatory cytokines and other proteins involved in the immune response.

The capsule, a linear polymer of γ-D-glutamic acid, is considered the other major virulence factor of *B. anthracis*. The capsule contributes to pathogenicity by enabling the bacteria to resist phagocytosis by macrophages, and thus evade the host immune defenses and promote septicemia. Additionally, the capsule is only very weakly immunogenic. As well as demonstrating the relative contributions of the above factors to strain virulence Coker *et al.* (2003) showed that the two plasmids were not singular but that their copy numbers, especially for pXO2 the capsular plasmid, predicted virulence.
Epidemiology

Anthrax is a disease known to man since ancient times and the first reports appear in the Bible (Exodus, Chapters 7 to 9) as the characteristics of the fifth and sixth plagues, which struck Egypt around 1491 BC, are identifiable with anthrax. Even writers in ancient times (Homer: Iliad I. 49-50, Trojan war, 1228 BC; Livy: Rome, 461 BC; Plutarch: 753 BC; Aristotle: History of animals, 333 BC; Virgil: Georgics, 32 BC) describe a livestock disease with the same characteristics as anthrax in their works (Blancou, 2003).

Between the late 19th and early 20th century anthrax was one of the infectious diseases with major mortality among domestic and wild animals. Probably the most serious incident occurred in 1923 in South Africa where in one year it killed between 30000 and 60000 animals (Sterne, 1967). Later with the appearance of the Sterne vaccine, of antibiotics, and the beginning of cost effective programs for prevention and control, the disease has lost its importance, taking on characteristics of sporadicity. Worldwide it is now an uncommon disease in much of Western Europe, Northern America and Australia, with exceptions in endemic foci in wild fauna in the African national parks (Hugh-Jones, 1999). In Canada it is enzootic in specific locations in the North-West Territories (Slave River Flats) and Alberta (Wood Bison National Park) (Nischi et al., 2002), and has the potential if control is relaxed to cause epidemics in the Canadian Prairie provinces, while in the U.S.A., the disease is a persistent threat in eastern North and South Dakota and North-West Minnesota, is enzootic in South-West Texas (Hugh-Jones, 1999) and suddenly ‘appeared’ in 2008 in South-West Montana where it had been unappreciated for decades. In Australia, anthrax is sporadic, although a sudden and severe epidemic occurred in Northern Victoria in 1997 (Turner et al., 1999). In Europe, the major enzootic areas are Greece, Spain, Turkey, Albania, France, and Southern Italy (Fouet et al., 2002; Fasanella et al., 2005), but it is essentially absent from Northern Europe.

While the incidence is generally decreasing worldwide it persists in certain countries; for example it is hyper-enzootic in Haiti and still enzootic in Bolivia, Mexico, and Peru. This follows from ineffective control programs. In contrast, vaccination programs in Belize, Nicaragua and Chile have resulted in good control. It is still absent from the Guianas. In Russia and in countries of the former Soviet Union, lack of effective control programs is evidenced by the high percentage of human cases, reflecting the inadequacies of both the public health systems and the veterinary services (Hugh-Jones, 1999). In Asia, anthrax is widespread in the Philippines, South Korea, Eastern India, and in mountainous zones of Western China and Mongolia; porcine anthrax is frequently reported in the highlands of Papua New Guinea. Africa remains severely afflicted, with major epidemic areas in wildlife areas such as Queen Elizabeth National Park (Uganda), Mago National Park Omo (Ethiopia), Selous National Reserve (Tanzania), Luangwa Valley (Zambia), Etosha National Park (Namibia), Kgalagadi Transfrontier Park (Botswana and South Africa), and Vaalbos and Kruger National Parks (South Africa). (Ebedes, 1976; Turnbull et al., 1991b; Hugh-Jones and de Vos, 2002). An anthrax-like disease has been found in wild non-human primates living in tropical rainforest, a habitat not previously
known to harbour *B. anthracis* (Leendertz *et al.*, 2004). It was characterized by an unusually high number of sudden deaths observed over nine months in three communities of wild chimpanzees (*Pan troglodytes resus*) in the Tai National Park, Ivory Coast. However, *Bacillus* strains associated with this outbreak were toxigenic *B. cereus* and not typical *B. anthracis*.

### Receptive animals

The sensitivity to anthrax varies considerably between the different animal species. In nature herbivores are particularly susceptible and omnivores and carnivores are moderately resistant but still succumb. The species most susceptible to infection, in descending order, are: cattle, sheep, horses, pigs, goats, and camels. Among herbivores, in addition to domestic species, including donkeys and mules, wild species are also susceptible and in particular zebras and the wild ruminants such as deer (Hugh-Jones and de Vos, 2002; Fasanella *et al.*, 2007) buffalo, gnu, gazelles, kudu, antelopes, and reindeer. Outbreaks have been reported in elephants, in some wild omnivores such as warthogs and bush-pigs, and among carnivores, as well as in the domestic dog and farmed mink. The disease has been reported in the national parks in Africa in wild dogs (lycaons, jackals), hyenas and the big felines (lions, cheetahs, leopards). Pigs and scavengers are more resistant to the disease than herbivores and they must ingest a large volume of bacilli to contract the infection. The greater susceptibility of bovines is related to their ease of getting infected and to the grazing habits of this animal. In the absence of upper incisors, they rip up the grass with their tongues, and this action facilitates the ingestion of soil, which is the main vehicle of bacillus spores.

### Transmission

Anthrax is not contagious from sick to healthy animals, but by the ingestion of spores dispersed into the environment. It’s widely believed that the vegetative forms of *B. anthracis* sporulate when exposed to oxygen. Under these assumptions it is assumed that in an intact carcass putrefactive processes should destroy almost all bacteria in a period of time ranging from 48 to 72 hours (Stein, 1947a). But rarely in nature are carcasses of dead animals left undisturbed (Fig. 4) by scavengers. Since the carnivores are less susceptible to the disease compared to herbivores, they can ingest larger quantities of infected meat and then spread spores in their feces (Pienaar, 1967; Turnbull *et al.*, 1989). During an epidemic in Basilicata in the summer of 2004, anthrax spores of the same genotype responsible of the epidemic were isolated from wild boar feces (not published). In rural areas scavenger birds such as ravens (*Corpus corax*) and vultures (various spp), can contaminate pastures or small bodies of water far from the original outbreak (Pienaar, 1967). Another form of pasture contamination occurs when necropsies are performed on-site by uninformed veterinarians or by farmers salvaging the hide by skinning a carcass. Even if the carcasses are deeply buried, the spores can be...
conveyed to the surface by the ground water or by land movements (Turner, 1999). In the past, washing waters from tanneries and wool mills were run off in water courses or used as fertilizer. Today outbreaks are associated with the use of soil contaminated forages produced on “champs maudit” contaminated land, and to products of animal origin such as meat and bone meals derived from the inadequate processing of infected carcasses. These products are very dangerous because they can cause unexpected outbreaks in conditions and situations epidemiologically very different from the classic episodes of telluric origin. The danger is that they can occur silently anywhere, even where anthrax is unknown, and at any time of the year.

Laboratory studies have shown, using mouse and guinea pig models, that stable flies Stomoxys calcitrans and Aedes aegypti and Aedes taeniorhyncus mosquitoes are able to transmit the infection. The percentage of transmission is very low (about 17% in the flies and 12% in the mosquitoes) but it is suspected that when the insect population density is high, they could be an important vehicle in the spread of the disease (Turell and Knudson, 1987). The role of tabanid Haematobia irritans in the spread of the disease was confirmed in two old scientific papers (Mitzmain, 1914; Morris, 1918). Lately Tabanid flies are considered to be of greater importance. (Kranelvede and Djaenodin, 1940; Rao and Mohiyudeen, 1958; Davies, 1983; Blackburn, 2006; Hugh-Jones and Blackburn, 2009)

**Pathogenesis**

The most common way of penetration by spores is via the digestive tract after the ingestion of spore contaminated feed, forages, and water. The ports of entry are micro-wounds that can be found in the mucous membranes of the mouth, pharynx and along the entire gastrointestinal tract. The infection can also occur through skin abrasions or skin lesions that may be caused by haematophagous insects (e.g. biting flies) acting as passive carriers or biological vectors. Although less frequent, spread is possible through the inhalation of dust containing spores. The severity of the disease depends on the sensitivity of the host, on the infectious dose, and on the route of penetration. Regardless of the route of penetration, it is considered that the spores of *B. anthracis* are carried by macrophages from the initial site of entry to the draining lymph nodes. The spores germinate, giving rise to vegetative forms that are capable of producing the main virulence factors: toxins and capsule.

Whatever the route of infection, it is believed that *B. anthracis* spores are transported by macrophages from the original site of introduction to draining lymph nodes and then enter the blood stream where they continue to multiply rapidly. The pathogenicity of *B. anthracis* depends on the quality of the capsular coat and the amount of toxins produced (Coker et al., 2003; Shoop et al., 2005) as well as on the sensitivity of the host species (Smith, 1973). In "Fisher 344" rats, the injection of the toxin causes death in about 30 minutes and a severe pulmonary oedema can be seen. Rabbits experimentally infected with *B. anthracis* show respiratory
symptomatology due to the intense action of the oedematous toxin on the lung. The leakage of blood from the nose is always just before or just after the death of the animal (personal observations).

Symptomatology.

Cattle and sheep

Bovine anthrax may be hyperacute or acute. In the hyperacute form animals are frequently found dead without the owner noting any obvious premonitory signs. This usually occurs at the beginning of an outbreak. In animals where clinical signs are observed, this will include fever, wheezing, congestion of mucous, muscle tremors, and convulsions. Death is sudden from shock and occurs within a few hours and is occasionally accompanied by the extravasation of blood from the nostrils, mouth, anus, and vulva. In the acute form the disease is characterised by septicemia, high fever (41°C-42°C), and tachypnoea, congested and haemorrhagic mucous membranes. Initially the animal may be excited, but this is followed by major depression. There is a loss of appetite and ruminal stasis. Pregnant cows may abort and those lactating may demonstrate a drop in milk production. Cutaneous oedema sometimes appears in the neck and ventral parts of the chest and abdomen. The animals die in 48-72 hours from shock.

In the sheep the fulminant form is most common; the animals can die in a few minutes with convulsions. Less acute affected animals stop eating, have high fevers, dyspnoea, muscle tremors and gnashing of teeth. Sometimes they will emit a reddish foam from the mouth shortly before dying.

Equines

The clinical manifestations and the course of the disease are almost always of an acute form with death occurring in 2-3 days. The disease develops with colic syndrome and septicemia associated with muscle tremors, sensory depression, a very high fever, cyanosis, tachypnea, and tachycardia.

Pigs

This species is more resistant and the disease is usually subclinical (Smith, 1973). It manifests as a localized swelling in the pharynx –the so-called "anthrax angina"– or in the intestine. If the location is in the throat there will be oedematous hot and painful swelling of the parotid region which may extend to the neck and chest, depression and fever, with anorexia or dysphagia, and cyanosis of the mucous membranes. There may be a profuse diarrhoea after an intestinal infection. When the lesions are severe, death occurs within 3-7 days. With only retropharyngeal or mesenteric lymph node inflammation the subject recovers. It seems that the nature of the contaminated feed can play an important rule since a fibrous abrasive feed can kill while the same spore dose in a soft feed will pass through the pig without apparent harm. (Ferguson, 1981)

Carnivores
These animals are fairly resistant but if affected they show signs of acute gastroenteritis and oro-pharyngitis which is due to ingestion of large volumes of infected meat. Usually recovery occurs spontaneously.

Pathology

Septicemic anthrax includes oedema, haemorrhage and necrosis (Gleiser, 1967). The lesions vary depending on the route of infection, the sensitivity of the host, and the virulence of the bacteria. Thus some animals show only signs of septicaemia, while in others just necrotic lesions occur (de Vos, 1994).

Cattle and sheep

In ruminants the disease is characterised by splenomegaly, bleeding and diffuse oedema predominantly in the connective tissues (Marcato, 1981). The carcass rapidly decomposes and swells (de Vos, 1994); rigor mortis is incomplete; and blood is dark red, is uncoagulable, and sometimes extravasates via natural openings (nose, mouth, anus, vulva). The blood clots are gelatinous because the normal blood coagulation processes are altered. This is accompanied by cyanosis and apparent mucosal bleeding, a gelatinous infiltration of the subcutaneous connective tissue, and congestion of the serosa, often with hemorrhagic petechiae (Contini, 1995), which collect a blood coloured liquid, particularly in the peritoneum, pleura and pericardium. Haemorrhages can be found throughout the internal organs. Sometimes small quantities of serum sweat from tissues of the neck and inguinal regions (Marcato, 1981). There may be blood mixed with urine in the bladder (Contini, 1995). The organ with the greatest changes is the spleen (de Vos, 1994), which has congestive-hemorrhagic tumefactions in the red pulp as a result of septicaemia. There is a significant increase in the volume of this organ and the capsule is tense; on dissection the pulp is red and black and the white pulp hard to see (Marcato, 1981). But splenomegaly is inconstant. The lesions may also affect the intestine; the internal mucosa is hyperaemic and full of punctiform haemorrhages. There are round tumefactions in the lymphoid tissue of the Peyer’s patches that are hemorrhagic-necrotic and ulcerative. The lesions can extend to the mesentry. Hemorrhage and oedema may be found in relation to the pharynx, larynx and lungs (Contini, 1995). Sometimes there are cases of cutaneous oedema because of a local infection (Marcato, 1981). Sheep are less resistant than cattle and therefore in these animals the disease develops faster.

Equines

In horses anthrax involves oedematous subcutaneous swelling of the neck, shoulders, chest, abdomen, and perineum (Sterne, 1959). The cutaneous oedema suggests a cutaneous reaction to bites from contaminated horseflies. When there is an infection of the pharynx or intestine from contaminated feed or forage there is often a diffuse hemorrhagic ulcerative enteritis. The regional lymph nodes are red and swollen with
yellowish areas of necrosis. Splenic lesions will be absent if the animal dies as a result of local reaction, without septicemia.

**Pigs**

In pigs the primary lesions are located in the pharynx and intestine as a result of the ingestion of infected meat leading to the formation of the so-called "angina anthrax". There is a hemorrhagic oedematous swelling of the mucosa and sub-mucosa of the pharynx, glottis, peripharyngial tissues, and of the subcutaneous connective tissue of the throat and neck (Henning, 1956). This is characterized by diphtheric membranes on the surface and deep, hemorrhagic, necrotic, grey-yellowish grey-brownish processes (Marcato, 1981). The regional lymph nodes – sublingual, retropharyngeal, sub-parotid – increase to several times their normal size. They are coloured dark red because of the adenopathy from the oedema, the iperemia, the haemorrhage and secondary necrosis (Ferguson, 1981). Anthracis pustules may form in the intestines and can be localised or diffuse, with hemorrhagic areas of inflammation affecting the wall of the intestine and corresponding mesentery. Only the mesenteric lymph nodes may be affected (Henning, 1956).

**Diagnosis**

Suspicion of anthrax arises from the observation of clinical symptoms, the anatomic-pathological findings and epidemiological data. The ecology of the bacterium limits the distribution of the disease that is almost always confined to the well defined territories, in which one can observe, with systematic cyclicity, sporadic outbreaks usually involving a few animals (one or two on average), but the frequency of which tends to increase during dry summers that follow very wet springs (Hugh-Jones, 1999). Less frequent and certainly more dangerous are introductive events that affect animals living in fixed stables which contract anthrax by eating contaminated food (usually forages) coming from high risk areas. This can and does happen in areas normally deemed free of anthrax and commonly in winter when livestock needs extra feed which will have to be purchased and may be contaminated. Thus, despite a careful epidemiological analysis, this can lead health professionals to misdiagnose suspect cases and consequently, the subsequent inappropriate management of infected carcasses that leads to an inevitable increase in the risk of infection in humans and other livestock. (Kreidl et al., 2006).

**Differential diagnosis**

In cattle anthrax should be differentiated from the following diseases:

- lightening strike and accidental electrocutions
- pasteurellosis
- piroplasmosis
- blackleg, malignant oedema and other clostridial diseases
However one should consider any disease causing sudden death or hemorrhagic septicemia. In horses we should consider colic syndromes, because of their symptomatology and infectious anaemia and dourine, because of the oedemas. However, in infectious anaemia, sublingual haemorrhages can be found.

**Laboratory diagnosis**

When taking a sample from a dead animal suspected of anthrax one needs to take precautions to prevent human infection, bacterial sporulation and a resulting environmental contamination. From live animals blood can be collected from the main superficial veins; while from dead animals, it can be taken from the peripheral veins, such as in the ear after removal of the auricle with a hot knife; in this way the wound is cauterized and the spilling of blood and contamination of the floor by spores prevented. The blood can be either on a cotton swab or in a vacutainer; the former is better. When using a cotton swab the blood should be allowed to dry, killing contaminants and encouraging any *B. anthracis* to sporulate. Putrefaction quickly destroys vegetative *B. anthracis*, which can therefore be difficult to isolate from the carcasses just 48 hours after death, especially in hot weather (Stein, 1947b). Traditionally ears are collected as they are convenient and far from the intestinal tract but a better sample are nasal turbinates which are well vasculated and therefore should have plenty of spores but with minimal tissue that is only little affected by putrefaction. One can also take serous liquids from oedematous areas and soft organs but these should be placed in leak-proof containers. At low temperatures (5°C - 10°C) it is possible to isolate the bacillus up to 4 weeks after death (Whitford, 1979). However, in reality the sample should be taken as soon as possible since decomposition leads to the rapid disintegration of the bacilli.

In the case of sheep or goats, one can send the entire carcass to the diagnostic laboratory in a leak proof container with a label indicating the suspected disease. This precaution must be taken even with single organs, such as the spleen or a collection of lymph nodes (in particular the prescapular node). When the carcass is too dehydrated, which can present diagnostic problems, one can collect soil from the ground under the animal that may have been contaminated by the leakage of blood and other body fluids from the natural openings and seepage. It should be noted that the longer an animal has been dead the smaller is the probability of getting a positive diagnosis, even with an experienced diagnostic laboratory.

**Microscopic test**

A preliminary examination with an unstained fresh blood smear will highlight the presence of rod like forms or typical "bamboo canes". The organisms are immobile and well capsulated. The slide may be fixed and stained with Gram stain and *B. anthracis* is violet. Preferably one can use Giemsa stain which stains the bacilli purple and the capsule a characteristic red mauve or with MacFadyean stain, which is blue methyl polychromatic and stains the capsule pink. Löffler uses methylene blue to which K₂CO₃ to 1% has been added (Turnbull, 1998). For *Bacillus anthracis* this leads to the metachromatic phenomenon with the bacterial
bodies stained blue while the capsule takes on a reddish colour. In the preparation of the slide one must take
care to pass the slide several times over the flame because the usual methods of fixing colours do not
inactivate the spores, which can represent a significant danger to the staff that will handle these microscopic
preparations. Anecdotally are stories of students getting cutaneous lesions from handling sharp-edged broken
blood smear slides that were decades old.

- Cultural test

*Bacillus anthracis* grows easily on normal agars, whether liquid or solid. Using a sterile loop the plates can
be sown with material from samples of blood, exudates, oedematous infiltrations, organs or parts of them
taken from infected or suspected animals. When one suspects the presence of spores in the material used in
the sample (wool, hair, leather, environmental samples) it is necessary to first incubate the material at 72°C
for 30 minutes to destroy contaminating bacteria, yeasts and moulds. It is always better to use a semi-
selective medium to isolate the bacterium. Moreover blood-containing media are preferable in comparison to
the often-used PLET or a Knisly agar, such as TSPB Agar, which is made highly selective against Gram-
negative bacteria by supplementation with trimethoprim (13.1 mg/L), sulfamethoxazole (20 mg/L) and
polymyxin B (30000 IU/L) (Tomaso et al., 2006). The plates are then incubated at 37 °C for 24 hours. If the
bacterium is present in the material used, white colonies will develop, 2-5 mm in diameter, of a pasty
consistency, and non-haemolytic. At a small magnification one can see long filaments folded several times
on their own that seem to have the appearance of the foliage of a jellyfish, the so-called Medusa's Head.

- Biological Test

It is usual with this kind of test to use particularly sensitive laboratory animals such as guinea pigs. The
inoculation of suspect material subcutaneously or intramuscularly is not recommended, especially when the
inoculate is full of secondary putrefactive bacteria. It is better to set up a test infection by coating the
material on an area of abdominal skin which had been previously shaved and scarified. This technique takes
advantage of the ability of *B. anthracis* to penetrate scarified skin, selecting it from the mixed microbial
flora. Rabbits die within 72 to 166 hours (Fasanella et al., 2009) and this depends on the virulence of the
different strains of anthrax and the number of organisms. However, after a few hours a gelatinous,
haemorrhagic oedema forms at the point of inoculation, which is then followed by all the other
characteristics of an anthrax lesion.

- Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is the method of choice as a parallel diagnostic test, whether performed
directly on clinical samples after non-selective enrichment of mixed cultures, or as a confirmation test for
suspect colonies. Additionally, PCR has become necessary for clarification of negative cultural results of
positive controls or when spiked agar plates are uninterpretable due to an overgrowth of *B. anthracis* by
haemolytic *Bacillus* spp. To confirm suspicious colonies, the DNA template for PCR can be extracted by
boiling resuspended colonies in TE buffer for ten minutes. To prepare DNA from a non-selective enrichment culture, a DNA Preparation Kit generally gives a better result. DNA can be prepared directly from spores by simple heating or autoclaving if very high numbers of spores (>10^6) are present. However, if only low numbers of spores are present or expected, as in the case of nose swabs or in secondary contamination scenarios, DNA preparation should be preceded by spore germination in culture. To identify virulent *B. anthracis* strains, and for the differentiation of non-virulent strains, the presence of both plasmid pXO1 (toxins) and pXO2 (capsule formation) must be confirmed. Some chromosomal targets of rpoB, S-layer protein genes and Ba813 very often lead to false-positive results from environmental samples (Papaparaskevas *et al.*, 2004), while *plcR* is able to differentiate *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis* (Easterday *et al.*, 2005).

- Molecular characterization

The full molecular characterization of *B. anthracis* was first completed with the Anthrax Genome Project, which in 2003 led to the publication of the genome of strain *B. anthracis* Ames type (Read *et al.*, 2003). For the scientific community this was a milestone, so that today, in Genbank there are 14 assembly, 5 complete and 4 incomplete sequence projects of 19 strains of anthrax. The genome of *B. anthracis* is composed of one chromosome with more than 5,000,000 bases and two plasmids, pXO1 with over 180,000 bases, and pXO2 with over 94,000 bases (Read *et al.*, 2003).

*B. anthracis* represents one of the most genetically homogeneous bacteria, with more than 99% of homologous nucleotide sequences. Searches made before 2001 showed small molecular variations among strains of different origin. In fact the complete sequence of the gene PagA (Protective Antigen) and the MLST (Multiple Locus Sequencing Typing) analysis of housekeeping genes revealed a few mutations (Okinaka *et al.*, 1999; Price, *et al* 1999). The revealed genetic crystallization is a direct consequence of its *modus vivendi*. In fact the vegetative form alternates with the spore form in an unpredictable frequency as a consequence of the regional ecology of the disease such that it may take years or even decades between infectious cycles. The evolutionary pressure is extremely low in the dormant (spore) stages of the cycle, remaining minimal during the active phase (vegetative form) because it is estimated that a maximum of 40 bacterial generations can follow (Keim *et al.*, 2004). Consequently *B. anthracis* evolution is temporarily reduced as the number of generations in a year are few when compared with *Escherichia coli* (Gutmann *et al.*, 1994) The precise molecular typing of strains of *B. anthracis* is crucial both for forensic analyses of biosecurity and bioterrorism events and for epidemiological investigations of natural outbreaks. The traditional methods are not very recommended for anthrax genotyping because they are unable to detect small genomic differences.

The genomic diversity is mainly the result of events during the evolution of the bacterium and genomic analysis must rely on molecular markers as polymorphic as possible with a high rate of mutation. The
anthrax genotyping methods currently in use test different types of markers in relation to the utility of the analysis.

The genotyping method, considered to have a low resolution, is the analysis of SNPs (Single Nucleotide Polymorphisms) and identifies point mutations in the genome. These markers have a good stability with a genomic mutation rate of $10^{-10}$. While there is a low rate of mutation some 35000 SNPs comprise the entire genome of anthrax (Pearson et al., 2004, Read et al., 2002). At present the opportunity to test all the identified SNPs in any isolate is technically difficult and financially expensive. However some studies have helped identify 12 canonical SNPs that are the most stable and homoplastic and can be used for phylogenetic investigations. Polymorphism analyses may be carried out using Snapshot or with real time PCR assays with TaqMan MGB probes (Van Ert et al., 2007)

The high resolution typing assay par excellence is that of Multiple Locus VNTR Analysis (MLVA) as it seeks to identify specific genomic regions known as Variable Number Tandem Repeat (VNTR). These regions of repeated DNA in tandem by their nature have a higher rate of mutation. The frequency of mutation of these markers in \textit{B. anthracis} is comparable to $10^{-5}$ with a high variability depending on the locus (Keim et al.,2004). This technique initially with 8 VNTR was able to identify 89 genotypes among 400 isolates from around the world (Keim et al., 2000), while the 15 VNTR assay increased this to 221 genotypes among 1033 isolates. The method has now been increased up to 25 loci (Lista et al., 2006), which allows an excellent discrimination. Technically, the VNTR are searched using capillary sequencers to analyse DNA fragments.

Lately high resolution assays were reported that examine markers called SNRs (Single Nucleotide Repeats), a sort of VNTR consisting of repeated sequences of poliA. Stratilo et al. (2006) through a bioinformatics analysis identified specific regions with a mutation rate of $10^{-4}$. Utilisation of these regions allows discrimination between organisms with the same MLVA pattern and thus allows sub-genotyping. The instability of these loci doesn’t make them homoplastic because back-mutations often occur. Their use can differentiate strains within the same outbreak or epidemic. A recent study has suggested the use of a panel of 4 SNR markers (Kenefic et al., 2008).

The described genotyping methods can be understood in a hierarchical way. The SNPs being at low-power of discrimination can be used for phylogenetic investigations. On the other hand VNTRs and SNRs have high discriminatory powers. The first for its high diversity and homoplasia is able to correctly define the genotype, while the latter searching for any signs of redundancy is considered suitable for identifying sub-genotypes. All the methods described are best performed by specialized laboratories experienced in molecular biology (Keim et al., 2004).

Vaccines
The veterinary vaccines currently used to fight anthrax are composed of spores from attenuated strains of *B. anthracis*. They are classified into two categories (Turnbull P., 1991):

- Live attenuated vaccines, capsulated and atoxigenic cap+/tox-; e.g., Pasteur vaccine.
- Live attenuated vaccines, not capsulated and toxigenic cap/-tox+; e.g., Sterne and STI vaccines (Turnbull, 1991a; Fasanella et al., 2001).

**Pasteur vaccine**

The Pasteur vaccine belongs to the first group and it is characterized by a plasmid pattern pX01/pX02+. The inactivation of *B. anthracis* is a function of the growth conditions. Grown at 42°C for 21 days it produces an attenuated strain, Pasteur vaccine type 1. It is not pathogenic for guinea pigs but it is pathogenic for mice. When grown at 42°C for 14 days it produces an attenuated strain that is more pathogenic than Pasteur vaccine type 1. It is pathogenic for both guinea pigs and mice, but is not pathogenic for rabbits. Pasteur vaccines should not produce toxic factors. Seroanalysis of goats vaccinated with Pasteur vaccine did not show the production of specific antibodies (unpublished data). Pasteur vaccines were used in the past, and they routinely produced a 3% mortality. Presently only a few countries still use this vaccine. In practice, the preparations used were “hot”, containing a mix of cap+/tox- and cap+/tox+ organisms, thus the mortality noted historically can be explained in this way.

**Sterne vaccine**

The Sterne vaccine represents another group of vaccines. It was produced for the first time in 1939, and was obtained by cultivating *B. anthracis* on a medium with 50% horse serum at 30% CO₂ atmosphere for 24 hours. It is very protective. This and similar vaccines are characterized by an elevated protective capacity and very low residual virulence. The attenuation of these strains is due to the loss of plasmid pX02 encoding the capsule synthesis; the spores germinate in the vaccinated animals and sufficient non-encapsulated vegetative forms germinate and produce toxic factors which are then neutralized by phagocytes allowing the synthesis of sufficient toxin to stimulate the protective immunity, but not sufficient to damage the host.

The animal vaccines that use Sterne strain 34F₂ are formulated with approximately $10^7$ spores per ml suspended in 0.5 ml 50% glycerine-saline. The protective effect of a single dose of strain 34F₂ vaccine is expected to last about one year and an annual booster is recommended for livestock in endemic areas. Horses and other equids can respond poorly and need two doses 4-8 weeks apart. South American camels and goats are very sensitive to Sterne and should be first vaccinated with a quarter-dose, followed 3-4 weeks later by a full dose; the best procedure is that they are vaccinated in their tail fold. The duration of the protection has never been systematically studied and is an area of research that needs to be studied in depth. Sterne vaccine is less expensive compared to the recombinant vaccines and actually it represents the best vaccine for routine anthrax control programs, especially if it is administered in the spring, to ensure the presence of protective immunity during the summer months, when most anthrax outbreaks occur. Spring vaccination
should provide adequate protection in those countries where livestock is grazed on high pastures during the
summer but are exposed when coming down to their winter pastures at a lower altitude.

**Third group of Bacillus anthracis vaccines: Carbosap strain**

The strain “Carbosap” was obtained from Prof. Cilli’s research group in the “Istituto Vaccinogeno of
Asmara”, during World War II. It was used from 1949 up to 2006 in Italy to immunize cattle and sheep and
then it was replaced by the Sterne vaccine. The PCR analysis using specific primers for the genes encoding
for PA, LF, EF and capsule showed the presence of both plasmids, pXO1 and pXO2 (Fasanella et al., 2001).
It has the same plasmid pattern of a pathogenic strain and shows no genetic difference in the DNA sequences
for the genes involved in the virulence (Adone et al., 2002). Carbosap vaccine is pathogenic for mice and
guinea pigs but it is apathogenic for rabbits.

**Emerging Vaccines**

A recent recombinant vaccine, whose preparation requires expensive procedures, could be considered a
useful vaccine in cases of emergency or during an anthrax epidemic when it is necessary to induce high
levels of protective antibodies in a very short time. Used in conjunction with long-acting antibiotics
recombinant vaccines have the capacity to produce a rapid response at the same time as infections are
neutralized. (Fasanella et al., 2008).

**Therapy**

Usually in most herbivores anthrax develops so quickly that it is not always possible to implement a
successful treatment.

The therapy is successful if it is applied as soon as possible after the animal is diagnosed to be ill, because it
has to prevent the production of toxins and antibiotics have no effect on toxins. In practice, therapy is
effective only in the early stages of the disease if it doesn’t have an hyperacute course. Antibiotic therapy in
humans is important. The current standard for anthrax inhalation post exposure therapy is ciprofloxacin twice
a day for 60 days (http://www.bt.cdc.gov/agent/anthrax/needtoknow.asp) (Food and Drug Administration.
Prescription drug products; Doxycycline and Penicillin G Procaine administration for inhalational anthrax
(post-exposure). Federal Register 2001;66:55679) (Friedlander et al., 1993). However in a study of 65
isolates of *B. anthracis* to determine the patterns of antimicrobial susceptibility it was found that one isolate
of *B. anthracis* was beta-lactamase positive and resistant to penicillin. All *B. anthracis* isolates were
susceptible to chloramphenicol, ciprofloxacin, clindamycin, rifampicyn, tetracycline and vancomycin
(Mohammed et al., 2002). In another study 22 *B. anthracis* isolates were tested for susceptibility to 27
antimicrobial agents by agar dilution. All isolates were sensitive to penicillins and did not produce beta-
lactamase. Although all isolates were sensitive to cefazolin, cephalothin, cephradin and cefoperazone, 19 isolates were resistant to cefuroxime, 18 to cefotaxime, 18 to ceftizoxime, 9 to ceftriaxone and 21 to ceftazidime. All isolates were also found to be sensitive to ofloxacin and ciprofloxacin (Doğanay and Aydin, 1991). Coker and colleagues found that in a cluster-selective collection of 25 isolates 3/25 were resistant to penicillin and 5 to cefuroxime. (Coker et al., 2002) However, unpublished results from testing some 1200 isolates showed that 3% to 6% were resistant to penicillin depending also on the region in the world the isolate came from. This applied to other antibiotics and to phage resistance. Antibiotic and phage resistance is not unusual, it is just not common.

There are many antibiotic options to eliminate an anthrax infection, but there are no therapeutic options to combat the LF-mediated toxemia and the tissue destruction during an ongoing infection or during the residual toxemia that persists even after the bacteria have been eliminated by antibiotics. Research on inhibitors of toxins has led to the identification of various substances whose action appears to be effective. Green tea extracts are rich in polyphenols and some of them are good inhibitors of LF in vitro and in vivo (Dell'Aica et al., 2004). The best one is (−)epigallocatechin-3-gallate (ECGC). Branched peptides have been found to inhibit both EF and LF binding to PA (Pini et al., 2008). A powerful inhibitor of EF is adefovir dipivoxil (Shen et al., 2004). Using a rabbit model we tested EGCG and adefovir alone or in combination with antibiotics to assess the possible therapeutic role of these potent inhibitors of both anthrax toxins. We found that they are protective only if they are given in the very beginning of the infection (Fasanella and Tonello, unpublished). This suggests that the role of toxins is critical for the initiation of pathology and that therapy with toxin inhibitors may not be promising (Fasanella and Tonello, unpublished). However, it has been demonstrated that a small molecule, hydroxamate LF inhibitor, can ameliorate the toxaemia characteristic of an active B. anthracis infection and might be a vital addition to our ability to combat anthrax. (Shoop et al., 2005). Finally, capsule-degrading enzymes favour the activity of neutrophils and other components of innate immunity offering a new approach that may be effective against antibiotic and/or vaccine-resistant strains. (Scorpio et al., 2008).

**Human anthrax**

Man is usually resistant to acquiring infection, but when infected may show three different clinical forms: the cutaneous, respiratory, and intestina forml.

The cutaneous form begins with the classic malignant pustule most often is localized to the face, neck, arms, hands or legs. Most frequently this involves specific high risk occupations; i.e. farmers, butchers, tanners, wool carders, shearers, and veterinarians. The most common exposure comes from skinnnig and butchering cattle sick or dead from anthrax. At the point of entry of the germ – a pre-existing scratch - there is a skin
redness which turns into a papule. Characteristically this lesion area is not painful. The surrounding area appears hyperaemic and oedematous. The papule develops into vesicles that spontaneously or from scratching break, and eventually it is covered with a black eschar. Sometimes the regional lymph nodes can be inflamed. Cutaneous anthrax is easily treatable with antibiotics of choice, but if a pustule is neglected it may evolve into a fatal septicemia. Some 10% of untreated cutaneous cases may die.

*Intestinal* anthrax results from the consumption of contaminated meat. Its symptoms include nausea, loss of appetite, vomiting, and fever followed by abdominal pain, vomiting of blood, severe diarrhea, lesions and soreness in the throat, difficulty swallowing, marked swelling of the neck and regional lymph glands. Intestinal anthrax results in death in 25 percent to 60 percent of cases (Beatty *et al*., 2003). The intestinal form occurs less frequent and occurs in those developing countries where food safety controls measures are de facto non-existent. A recent case occurred in an hamlet in Vietnam, where two families shared beef from their two cows, which had died of an unknown cause. Those eating the meat started vomiting, complaining of stomachache and suffering swollen legs. Two of them died in coma after being hospitalized ([http://www.promedmail.org](http://www.promedmail.org)). Thorough cooking will kill the vegetative cells and prior exposure does provide some immunity so the attack rate in any incident is variable.

The *respiratory or pulmonary* form is the major cause of atypical haemorrhagic pneumonia starting with flu-like symptoms, characterized by fever, muscle pains, coughing, red nose, and bloody sputum. Untreated cases are fatal. A study on the first ten confirmed cases of inhalational anthrax caused by an intentional release of *B. anthracis* in the United States showed that the median incubation period from the time of exposure to onset of symptoms was 4 days (range 4 to 6 days). Symptoms at initial presentation included fever or chills, sweats, fatigue or malaise, a minimal or non-productive cough, dyspnoea, and nausea or vomiting. All ten patients had abnormal chest X-rays; abnormalities included infiltrates, pleural effusion, and mediastinal widening. Computer tomography of the chest was performed on eight patients, and mediastinal lymphadenopathy was present in seven (Jernigan *et al*., 2001). Forty-one cases of documented inhalational anthrax from the Sverdlovsk epidemic of 1979 showed that the lesions that were the most severe and apparently of the longest duration were in the mediastinal lymph nodes and mediastinum (Meselson *et al*., 1994). There and elsewhere, peripheral transudate surrounded a fibrin-rich oedema; necrosis of arteries and veins was the most likely source of large haemorrhages displacing tissue or infiltrating tissues, respectively; and apoptosis of lymphocytes was observed. Respiratory function was compromised by mediastinal expansion, large pleural effusions, and haematogenous and retrograde lymphatic vessel spread of *B. anthracis* into the lungs with consequent pneumonia. The central nervous system and intestines manifested similar haematogenous spread, vasculitis, haemorrhages, and oedema. (Grinberg *et al*., 2001.)

**Considerations**
B. anthracis is known for its rapid proliferation and dissemination in receptive hosts but we still know very little about its ability to replicate outside the host. Recently it was shown that B. anthracis can survive as a saprophyte outside the host. The data suggest that horizontal gene transfer in the rhizosphère of grass plants may play a role in the evolution of the B. cereus group species (Saile and Koehler, 2006). These data were obtained under experimental but sterile conditions and we do not know if this actually occurs in nature.

It is clear that in the environment, where conditions are less favorable to the survival of vegetative forms, B. anthracis spores are one of the most advanced forms of resistance known in nature. In the Kruger National Park (Africa) B. anthracis spores have been isolated from animal bones estimated to be about 200 years old (Smith et al., 2000). The calcium cation is the most important component in the process of germination or in the maintenance of latency and hence the calcareous soils, rich in calcium and typically with an alkaline pH contribute to the formation of much more resistant spores. The persistence of spores in the ground is essential but not in itself sufficient to give rise to new anthrax outbreaks. Van Ness, whose studies represent a milestone in understanding the ecology of anthrax, noted that the outbreaks of anthrax develop mainly during the dry months that follow a prolonged period of rain. These climatic aspects and the fact that the spores are characterized by a high floating capacity suggest that water plays an important role in the ecology of the bacterium. Rainwater, having washed away the surrounding ground, tends to stagnate in the low lying parts favoring the concentration of spores. This sequence of events encourages the adhesion and the distribution of spores on soil humus, so the chances to infect herbivores increase. However it takes time and special natural events to create sites of concentrations of spores which can cause new infections in grazing animals (Van Ness, 1971). Most of the existence of B. anthracis is held in the ground as spores until the ideal conditions are created for its reproductive cycle that occurs in a different habitat, primarily domestic and wild ruminants. Nature provides few opportunities to the bacterium for its replicative cycle, and the development of an exceptional pathogenicity is the effective strategy aimed to significantly increase the probability of success against the host’s immune mechanisms. Rapid intense multiplication by the vegetative cells quickly take the host to death.

Although many of the new generations of bacteria will be neutralized by putrefactive processes, a good part survives and spreads in the surrounding soil as spores, ensuring the standard of environmental density of the bacteria that is an essential condition for the continuation of the species. In summary, the few cases of anthrax that occur each year are merely the result of a natural ecological balance that seeks through these extraordinary events simply to promote the maintenance of a bacterial species that otherwise would have been extinguished some time ago.

Animals from areas free of anthrax and moved to risk areas are more susceptible to disease. The project to reintroduce red deer (Cervus elaphus) into some nature reserves in Basilicata (Southern Italy), is facing major obstacles just because of the sensitivity of this particular animal species to anthrax infection (Fasanella...
et al., 2007). At this time we do not know if this is related to the specific animal species or to a lack of natural antibodies, but it is certain they come from ecosystems in which anthrax was not present. In nature there are no natural self-destructive behaviors and every living being has evolved its own strategies for survival, not only in terms of preservation of their species but also in that of its ecosystem. So could we not hypothesize that B. anthracis has a protective role in the delicate balance of its ecosystem, somehow preserving animal species that are an integral part of that particular area from the possible risk of extinction from infectious diseases introduced by previously unknown animals from different environments? (personal opinion)

The control and eradication programs of this disease involves livestock vaccination and the correct management of infected carcasses. Incineration in situ is the best method to reduce the contamination level of soil, but it is not always possible. A major constraint is the low budget that civil authorities assign for the control of this disease. This makes it impossible to use the most cost effective technologies for control programs. A global responsibility should oblige local governments to guarantee sufficient budgets for control and eradication programs of this disease and not just for emergency situations.

Conflict of Interest Statement

None.

References


Figure 1: *Bacillus anthracis*. Vegetative form, Gram stain

Figure 2: *Bacillus anthracis*. Spore form, Shöeffer Fulton stain

Figure 3: *Bacillus anthracis* colonies in semi selective agar (TSPB). It can notice the typical aspect with indented margins.

Figure 4: *Bacillus anthracis* grown in liquid agar. The vaccine strain Sterne 34F2 grows mudding the medium, while the pathogenic strain creating flocculus.

Figure 5: Red deer found after a long time from Its death. The signs of wild carnivores are well evident