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Anthrax

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1. Introduction

1.1 Definition

Anthrax is a non-contagious infectious disease hitting a high range of animal species including humans, although the animals that are most susceptible are domestic and wild ruminants. The bacterial agent is *Bacillus anthracis* whose main characteristic is to form spores that can survive outdoors for several decades. Anthrax in susceptible animals generally has a fatal evolution characterised by sudden deadly bleeding from natural openings. In humans, the disease develops in three forms depending on the route of penetration of the bacterium: cutaneous (non-fatal), pulmonary and gastrointestinal. Recently a fatal form was reported characterised by a subacute evolution in drug users as a result of injection of drugs contaminated with anthrax spores. Due to its high capacity to maintain its viability and pathogenicity and for low cost production, B. *anthracis* is considered one of the pathogen agents of greatest interest for use as a bacteriological weapon in bioterroristic attack.

1.2 History

Anthrax is a disease known since ancient times. Probably the first record of the disease can be found in the Bible in the Book of Exodus Chapter 7-9. It is thought that the V plague that struck Egyptian people is a disease that has clinical features very similar to anthrax. Another allusion to a disease very similar to anthrax is made by Homer in the Iliad when he speaks of a "burning wind of plague". Then Hippocrates (5th century B.C.), using the Greek word for "coal", defined a disease characterised by skin dark lesions and fluid blood.

But it is the Roman poet Virgil in his Georgics who described anthrax in detail and for the first time hypothesized on the transmission from animals to humans, suggesting attention to the ongoing slaughter of animals with the disease.

Anthrax has been for a long time the main and most feared disease among animals and the epizootics of anthrax have been responsible for real massacres of animals up to the 19th century. A serious outbreak in the mid-18th century seems to have destroyed half of the entire population of sheep in Europe. Chaber in 1780 described in detail the disease in animals and over the same period Barthelemy showed the transmission in healthy animals by the inoculation of infected blood. The appearance of zoonotic anthrax had been widely highlighted by the fact that human cases of this disease increased during the epidemic

among animals. Maret and Fournier in 1769 studied this aspect of the disease due to the fact that people who came into contact with sick animals often developed skin ulcers, which if not properly treated could lead to a fatal septicaemia. Pulmonary anthrax had long been known, especially as the "wool-sorter disease". If untreated, it rapidly leads to death. As for gastro-intestinal anthrax, due to consumption of meat from diseased animals, it caused considerable human fatalities simultaneous with anthrax epidemics in animals. Thus, for instance, in 1613, the disease caused 60,000 human fatalities in Southern Europe (Schwartz, 2009). In 1958, the WHO estimated the annual incidence of human cases of anthrax worldwide to be between 20,000 and 100,000.

Anthrax is not only a disease of the past. It is still with us today, not only as a potential weapon for bioterrorists.

In developed countries, due to the application of adequate prophylactic measures, it is sporadic. In contrast, in developing countries, anthrax may still represents a major problem, for animals as well as for human (Hugh-Jones, 1999; Hugh-Jones & Blackburn, 2009). A massive outbreak occurred in Zimbabwe during the period 1978–1980, which caused 9,711 human cases with 151 deaths (WHO, quoted by Turnbull). More recent examples are the epidemics in Kyrgyzstan and Zimbabwe. In the former, large but unknown numbers of animal cases were accompanied by at least 50 human cases in 2008. In Zimbabwe, in 2008, anthrax added its toll to the severe epidemic of cholera in a totally disorganised country where actual numbers of disease victims are difficult to ascertain; WHO reported some 200 human cases with eight confirmed deaths. In Bangladesh in 2010 there were 104 animal cases of anthrax and 607 associated human cases from contact with contaminated meat from sick livestock (Fasanella et al., 2011).

2. Characteristic of *Bacillus anthracis*

2.1 Aspect

B. anthracis belongs to the family of Bacillaceae and has a rod-shape (long 3 - 6 µ and wide 1 - 1.5 µ). It is motionless and aerobic. Often there are different elements assembled in a chain. In preparations fixed and stained the extremities appear at right angles or enlarged and the surface of contact between the individual elements is concave, similar to the epiphysis of a bone, and that gives them a particular look similar to "bamboo canes" (fig. 1). Bacilli in the animal organism are surrounded by a clear capsule that is usually lacking in culture media and is considered as a defence by the forces of the germ-bacterial organism. Sometimes the *B. anthracis* undergoes lysis phenomena: the capsules are intact while the inside contains only remains of the bacillary body, some are completely empty capsules (shadows). This is especially true in the material in the process of putrefaction. Outside the body and with temperatures between 14°C and 42°C (optimum between 21°C and 37°C) *B. anthracis* will sporulate. 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 CO_2, a condition that occurs in infected putrefacting carcasses.

2.2 Staining

B. anthracis is coloured with all the aniline dyes. It is Gram-positive. In blood or organ smears stained with methylene blue Löffer, the bacillary body is coloured in blue and purple

capsule (sometimes only purple spots are observed, probably due to the material of the capsule: reaction of Mc. Fadyean).

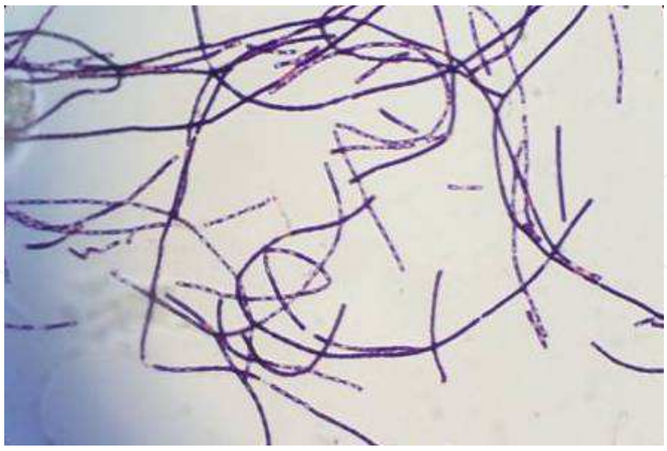

Fig. 1. Gram stain of *Bacillus anthracis* vegetative form from a colony growth on agar TSP 5% sheep blood. It is evident the typical bamboo-shaped filaments

2.3 Cultivation

B. anthracis grows well on ordinary culture media under aerobic or microaerofilia, at temperatures between 12°C and 44°C, but optimal growth occurs around 37°C and at a pH of 7.0 to 7.4. In tryptose broth there is a flocculation and then it forms a silky deposit. Colonies on plates form a magnificent plot called caput medusae (phase R or rough) (fig. 2). For this reason is generally believed that the R phase of B. *anthracis* is the normal and virulent one, while the attenuated (vaccine germs) grow mostly in S phase. This would be an exception, as with other microbial species S phase is the normal and virulent phase. But it seems that the physiological condition for the growth of anthrax bacilli, including the presence of carbon dioxide concentration of at least 5% (which occurs in the alveoli), permits

Fig. 2. Colony of *Bacillus anthracis* growth on TSP agar 5% sheep blood

the germs to grow in an S virulent phase (mucosal aspect of colonies). Probably the growth in the R phase, which occurs in an ordinary atmosphere, would be a temporary phenomenon of adaptation.

2.4 Resistance

Vegetative forms are not very robust and they are inactivated within 30 minutes at 60°C - 65°C (Turnbull, 1998), but its spores are very resistant. The action of direct sunlight is significant as ultraviolet rays will inactivate them in a few hours, however the spores that live a few inches deep in the soil will remain active for years. In fertiliser prepared so that the temperature reaches over 60°C (aerated compost, rich in horse faeces) spores are killed in a few days. In the cold and salting samples spores resist for a long time: frozen meat and skins remain virulent for years and the same is true for dried skins. The spores are destroyed only after ten minutes of boiling temperatures, they are destroyed in 20 minutes in an autoclave set at 121°C. 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 six hours or at 4% after a contact of at least two hours. The spores are destroyed by 5% phenol and mercury chloride, and 1% solutions of caustic soda and potash.

3. Ecology of anthrax

Anthrax spores survive best in soils rich in organic matter and calcium. 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). Saile and Koehler (2006) have demonstrated that spores will germinate and establish stable populations of vegetative cells in the rhizosphere of fescue (*Festuca arundinacea*) grass in the laboratory in an otherwise sterile environment. In natural circumstances the vegetative cells are fragile and die even in simple environments, such as water or milk (Turnbull et al., 1989). In conclusion it seems that soil encourages sporulation, not germination, and this would explain why vegetative bacilli are not found in nature. Van Ness (1971) defined the "incubator areas" as depressions which collect water, dead vegetation, calcium and other salts washed in from the surrounding slightly higher ground and thus provide a medium suitable for germination and multiplication. However, this hypothesis was never confirmed by scientific study. It has been proposed that rainy water may collect and concentrate spores in 'storage areas' (Dragon & Renie, 1995). Spores have a high surface hydrophobicity and so could be carried during a rain runoff in clumps of humus and organic matter to collect and concentrate in standing pools or puddles. As they have a high buoyant density, this would result in them and their organic matter clumps remaining suspended in the standing water to be further concentrated as the water evaporated. Thus theoretically 'storage areas' may collect more spores from extended areas to reach increasing spore concentrations over time and be lethally available to potential incidental grazing hosts. Most *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 takes the host to death. Although many of the new generations of bacteria will be neutralised by putrefactive processes, a good part survives and spreads into 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. It is widely believed that the vegetative forms of *B. anthracis* tend to 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 by scavengers. Spores will survive passage through the scavenger's intestinal tract, but vegetative cells will not. Anthrax spores were recovered from approximately half of the faeces from jackals (*Canis mesomelas*), vultures (*Gyps africanus, Torgos tracheliotus, Trigonoceps occipitalis*) and hyaenas (*Crocuta crocuta*) collected in the vicinity of carcasses in the Etosha National Park, but not at a distance; the faecal spore density was extremely variable (Lindeque and Turnbull, 1994). Insects, primarily necrophilic and haemophagic flies, have been associated in the spreading of anthrax spores. Fasanella et al. (2010) demonstrated that, under experimental condition, *Musca domestica* can spread the bacterium and additionally that *B. anthracis* is able to germinate within their intestines.

4. Toxic factors of *B. anthracis*

The pathogenic action of *B. anthracis* is closely linked to the following two plasmids:

- pXO1, 182 Kb, which contains the genes encoding the three anthrax protein factors: the oedema factor (EF), the lethal factor (LF) and the protective antigen (PA);
- pXO2, 96 Kb, which contains the genes encoding the biosynthesis of the capsule (Uchida et al., 1997).

The results of a study demonstrated that *B. anthracis* virulence is related to clonality (as indicated by MLVA genotype cluster) and pXO1 and pXO2 copy number (Cocker et al., 2003).

The capsule is a linear polymer of D-glutamic acid which plays an important role in the ability of anthrax to resist phagocytosis by macrophages. The exact mechanism by which this occurs, however, is still unknown. In contrast, the three protein factors have been, and still are, the object of much attention. Interestingly, the idea that the bacterium could secrete a molecule involved in pathogenesis was mentioned by Pasteur as early as 1877. Pasteur noted that filtrates prepared from the blood of diseased animals induced the agglutination of red cells in blood from healthy animals. Smith and his associates showed the complex to be composed of the three protein factors mentioned above: PA (83 kDa), EF (89 kDa) and LF (90 kDa). Independently, these three factors are innocuous. Intravenous injection of PA + LF, however, provokes death, whereas intradermal injection of PA + EF produces oedema in the skin. In the early 1990s, Singh et al. discovered that PA was the component involved in the specific binding of LF and EF to the target cell, as well as in the transport of these virulence factors into the cell (Singh et al., 1991).

Contrary to earlier studies suggesting that the toxins were responsible for death (Keppie et al., 1955; Smith et al., 1955), recent research indicates that their primary targets are cells of innate immunity that would otherwise impair anthrax multiplication (Tournier et al., 2009) . They do so by altering the cyclic adenosine monophosphate (c-AMP) and mitogen-activated protein kinase (MAPK) signalling pathways essential for the activation of immune cells. In brief, the two anthrax toxins derive from the combination of three different proteins: PA, EF and LF. PA binds to two cell surface receptors, the tumour endothelium marker 8 (TEM8) and the capillary morphogenesis protein 2 (CMG2), both of which are widely expressed on many cell types, including immune cells (Collier & Young, 2003; Scobie & Young, 2005). The proteolytic release of the C-terminal domain (20 kDa) of PA results in spontaneous oligomerisation of truncated PA (PA63) into heptamers, which bind EF and LF. The (PA63)7–EF and the (PA63)7–LF complexes enter rafts and – after endocytotic uptake – are transported to late endosomes, whose low pH induces a conformational change of the complex, with the insertion of a part of PA into the membrane and the translocation of EF and LF into the cytosol. EF is a calmodulin-dependent adenylate cyclase (Leppla, 1982) which creates a gradient of cAMP with a high concentration in the perinuclear area, whilst LF is a metalloprotease which cleaves most isoforms of MAPKKs (MEKs) throughout the cytosol (Vitale et al., 2000). This does not exclude the possibility that it may act on other cytosolic proteins as well, a possibility raised in recent reports suggesting that LF acts on the inflammasome (Boyden & Dietrich, 2006; Muehlbauer et al., 2007). MEKs are part of a major signalling pathway linking the activation of membrane receptors to the transcription of several genes, including those encoding pro-inflammatory cytokines and other proteins involved in the immune response.

5. Epidemiology

Knowledge of the disease, the agent, the transmission, the development of a vaccine and especially understanding that a relevant rule in the control of anthrax is the removing of infected carcasses from the environment to reduce the process of spore production, has contributed to the almost complete disappearance of anthrax.

In agricultural areas of industrialised and rich countries, the sporadic outbreaks of anthrax still tend to occur where in the past infected animals were buried or leather industry waste was collected. More frequently, outbreaks are reported that develop as a consequence of the introduction of contaminated feed. Probably the most serious incident occurred in 1923 in South Africa where in one year it killed between 30,000 and 60,000 animals (Sterne, 1967). Though 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 form epidemics in the Canadian Prairie provinces, while in the US. 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 not been recorded . 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 essentially absent from Northern Europe.

While the incidence is generally falling 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 programmes. In contrast, vaccination programmes 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 programmes 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., 1991; Hugh-Jones and de Vos, 2002). An anthrax-like disease has been found in wild primates living in tropical rainforests, a habitat not previously known to harbour *B. anthracis* (Leendertz et al., 2004) and characterised 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*.

6. Receptive animals

Under natural conditions the animals that are more susceptible to anthrax are ruminants, both domestic (cattle, buffalo, sheep, goats, camels, etc.) and wild (deer, roe deer, elephant, etc.). Horses are also receptive and pigs to a lesser extent.

Horses in natural conditions are less receptive to anthrax than cattle when the infection is transmitted via food, probably because they are monogastric and the spores ingested with food are quickly neutralised by the acid chloride present in the stomach. In cattle, however, before arriving in the stomach the spores make a long trip and this favours their implantation. On the contrary in anthrax infection transmitted through the skin, horses seem to be more sensitive because in the past, when the Pasteur vaccines were used, vaccination accidents were more frequent in the horse compared to ruminants. In anthrax outbreaks, because of the activity of biting flies, the value of horses affected/horse population is higher than the value of ruminants affected/ruminant population.

Carnivores are sick only exceptionally while birds are refractory.

Humans contract the infection almost always from infected products of animal origin.

7. Transmission

Anthrax ordinarily is a disease characterised by indirect transmission by means of materials (feed, straw, water, etc.) that have been polluted with spores.

As for the great resistance of the spores, polluting materials retain their infectivity for several years. It follows that in the pastures where dead animals or their residues have been abandoned the spores are durable and new infections happen when other animals graze or eat forages coming from these fields (telluric origin).

However, if the carcass was buried at shallow depth, the spores of anthrax can easily be brought to the surface of the ground thanks to an elevation of the waterbed and by movement of the earth due to the activity of earthworms and snails.

Another danger of infection is given to waste waters of tanneries where skins of infected animals are worked. This water often ends up in the irrigation canals and when the water flows out very slowly (e.g. stagnant) it leaves anthrax spores and other material on the vegetation. The import of food, wool, bristles, etc. from high risk anthrax areas are frequently the cause of the spread of infection. The infection can also be spread by animals, being naturally resistant to infection, that distribute anthrax spores in the faeces ingested with the food. Additionally, in cases of carnivores (dogs, foxes, vultures) that eat infected meat, outbreaks of anthrax have spread to distant points.

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). Recently Blackburn et al. (2010) isolated *B. anthracis* from flesh-eating flies and demonstrated the importance of these kinds of insects with a wildlife anthrax outbreak in North America and the potential role in anthrax epizootics. Moreover, the hypothesis that blood-sucking insects such as tabanids (gadflies or horse-flies) can play an important role in spreading diseases among livestock and other animals is widely accepted (Krinsky, 1976) (fig. 3).

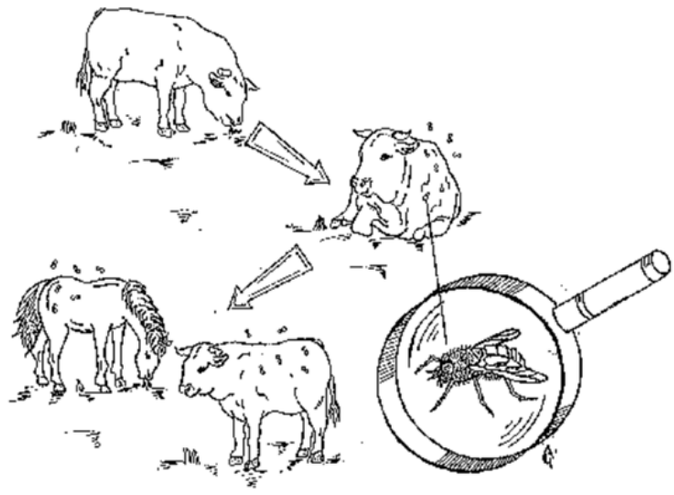

Fig. 3. Circulation of anthrax by means of horseflies (drawing by Gabriella Abbatangelo)

Anthrax human infection is rare in developed countries. However, recent outbreaks in the US and Europe, and potential use of the bacteria for bioterrorism have focused interest on it. Furthermore, while anthrax was known to typically occur as one of three syndromes related to the site of entry (i.e. cutaneous, gastrointestinal or inhalational), a fourth syndrome

including severe soft tissue infection in injectional drug users is emerging. However, the 2010 anthrax epidemic in Bangladesh, where 607 associated human cases were registered from contact with contaminated meat from sick livestock, underlined that anthrax has the potential to be a serious zoonotic disease in low income countries where there are few resources for an optimal infectious diseases control system in humans or livestock. It underscored the high risk to humans when exposed to infected animals through slaughtering and butchering.

8. Pathogenesis

The most common way of penetration by spores is via the digestive system 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 rapidly multiply. The pathogenicity of *B. anthracis* depends on the quality of the capsular coat and the amounts of toxins produced (Coker et al., 2003; Shoop et al., 2005) and on the sensitivity of the host species (Smith, 1973). In Fischer 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).

9. Anthrax in animals

The incubation period of the disease under natural conditions varies from one to 14 days, but usually three to five days.

9.1 Ruminants

In cattle, the symptom picture is quite variable. Some animals suddenly fall down and die in few minutes, without having presented any symptoms (fig. 4). Other times the death occurs after one to two days (rarely three to five) and the disease is characterised by the following symptoms: rapid pulse and respirations, anorexia, decrease or cessation of milk secretion, cyanotic mucous membranes, colic, outflow bleeding from the body's natural openings and oedematous swelling under the skin (especially in the neck, chest and belly).

Fig. 4. Hyperacute form of anthrax in cattle

These events are always accompanied by high fever (41°C and beyond) that tends to settle very early. In farms with active outbreaks it is possible detect the sick animals by measuring body temperature before clinical symptoms appear. In sheep and goats most of the time evolution is hyperacute. The animals are suddenly struck by dizziness, staggering, falling to the ground and die in a few minutes with leakage of blood from the body's natural openings.

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, uncoagulable and sometimes extravasates via natural openings (nostrils, 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 haemorrhagic petechiae (Contini, 1995), which collect a blood coloured liquid, particularly in the peritoneum, pleura and pericardium. But 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 also 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-haemorrhagic tumefactions in the red pulp as a result of septicaemia. There is a significant increase in the volume of this organ and the capsule tense; on dissection, the pulp is red and black, and the white pulp hard to see (Marcato, 1981). Splenomegaly, however, 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 plaques that are haemorrhagic-necrotic and ulcerative. The lesions can extend to the mesentery. Haemorrhage and oedema may be found in relation to the pharynx, larynx and lungs (Contini, 1995). Sometimes there are cases of cutaneous oedema because of local infections (Marcato, 1981). Sheep are less resistant than cattle and therefore for them the disease develops faster.

9.2 Equines

The clinical manifestations and the course of the disease are almost always of an acute form with death occurring in two to three days. The disease develops with colic syndrome and septicaemia associated with muscle tremors, sensory depression, a very high fever, cyanosis, tachypnoea and tachycardia. 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 haemorrhagic 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 septicaemia.

9.3 Pigs

This species is more resistant and the disease is usually subclinical (Smith, 1973). It manifests as a localised swelling in the pharynx – the so-called "anthrax angina" – or in the intestine. There may be a profuse diarrhoea after an intestinal infection. When the lesions are severe, death occurs within three to seven days. It seems that the nature of the contaminated feed can play an important role 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). With 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 "anthrax angina". There is a haemorrhagic oedematous swelling of the mucosa and sub-mucosa of the pharynx and glottis, of peripharyngial tissues, and of the subcutaneous connective tissue of the throat and neck (Henning, 1956). It is characterised by diphtheric membranes on the surface and deep, haemorrhagic, 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). Anthrax pustules may form in the intestines and be localised or diffuse, with haemorrhagic areas of inflammation affecting the wall of the intestine and corresponding mesentery. Only the mesenteric lymph nodes may be affected (Henning, 1956).

9.4 Carnivores

These animals are fairly resistant, but if affected, they show signs of acute gastroenteritis and oro-pharyngitis due to ingestion of large volumes of infected meat. Usually it heals spontaneously.

10. Anthrax in humans

10.1 Cutaneous form

More than 95% of all naturally occurring *B. anthracis* infections worldwide are cutaneous. This form of anthrax is associated with the handling of infected animals or contaminated items such as meat, wool, hides, leather or hair products from infected animals (Lucey, 2005). The majority of cutaneous anthrax lesions develop in exposed areas such as the face,

neck, arms and hands. The lesion begins as a small, often pruritic papule that quickly enlarges and develops a central vesicle or bulla, which ruptures or erodes, leaving an underlying necrotic ulcer. Another characteristic that is firmly adherent is black eschar developing over the surface of the ulcer, however, the risk for person-to-person transmission of cutaneous anthrax is very low (Heyworth et al., 1975). The incubation period for cutaneous disease is reported to be five to seven days (range: one to 12 days) (Carucci, 2002). However, during the 1979 Sverdlovsk outbreak, cutaneous cases reportedly developed over up to 13 days after the aerosol release of spores (Meselsen et al., 1994) and an outbreak in Algeria was reported with a median incubation period of 19 days (Abdenour et al., 1987).

10.2 Gastrointestinal form

Gastrointestinal anthrax typically occurs after eating raw or undercooked contaminated meat, although spores consumed through any route, including spores that are inhaled and subsequently swallowed, can result in gastrointestinal anthrax. The intestinal form develops when spores infect the gastrointestinal tract epithelium after consumption of undercooked, contaminated meat. Signs and symptoms range from subclinical gastrointestinal disturbances to clinical illness with nausea and vomiting, fever, anorexia and abdominal pain and tenderness, and can progress to haematemesis and bloody diarrhoea. Abdominal distension with voluminous, haemorrhagic ascites might be present. The disease might progress to septicaemia and toxaemia, cyanosis, shock and death (the incubation period for gastrointestinal disease is estimated to be one to six days; the case-fatality ratio is unknown, but is estimated to range from 25% to 60% (Sirisanthana & Brown, 2002; Kanafani et al., 2003; Ndybahinduka et al., 1984; Beatty et al., 2003).

10.3 Pulmonary form

Inhalation anthrax is a systemic infection caused by inhalation of *B. anthracis* spores. This form of the disease results from the inhalation of aerosolised *B. anthracis* spore-containing particles that are ≤5 microns (Druett et al., 1953). Spore-containing aerosols can be generated through industrial processing or work with spore-contaminated animal products such as wool, hair or hides; by laboratory procedures such as vortexing of cultures or as a result of the intentional release of aerosolised spores. Early studies of inhalation anthrax demonstrated that inhaled spores are phagocytosed by macrophages in the lungs and transported to the pulmonary-associated lymph nodes where germination and vegetative growth occur, followed by bacteraemia and dissemination to the rest of the body (Lyncoln et al., 1964; Henderson et al., 1956; Ross, 1957). Initial signs and symptoms of inhalation anthrax are non-specific and might include sore throat, mild fever and muscle aches; these symptoms might initially be mistaken for an upper respiratory infection (Temte & Zinkel, 2004; Lucey, 2005). Approximately two to three days later, infected patients generally become progressively ill as respiratory symptoms develop, including severe dyspnoea and hypoxaemia and the disease progresses with development of hypotension, diaphoresis, worsening dyspnoea, shock, cyanosis and stridor (Holty et al., 2006). Chest radiography often reveals the characteristic widened mediastinum (Jernigan et al., 2001; Lucey, 2005).

Case-fatality ratios of 86% and 89% were reported after the 1979 Sverdlosk outbreak in the former Soviet Union and in the United States during in the 20th century, respectively (Meselsen et al., 1994; Brachman, 1980; Brachman & Fridlander, 1994). During the bioterrorism events of 2001, the case-fatality ratio for patients with inhalation anthrax treated in intensive care units was 45% (five of 11 cases) (Jernigan et al., 2002).

10.4 Bacteraemic dissemination and meningitis

After infection at the primary cutaneous, gastrointestinal or inhalation site, lymphatic and haematogenous proliferation of anthrax bacilli can result in dissemination to other organs and organ systems (i.e. systemic anthrax). Massive septicaemia with 107 to 108 bacteria per millilitre of blood and toxaemia can develop, systemic effects, including high fever and shock, develop quickly and death usually follows rapidly (Dixon et al., 1999). Anthrax meningitis has been reported with all three clinical forms of anthrax and likely results from haematogenous spread across the blood-brain barrier, generally presenting as haemorrhagic meningitis. Anthrax meningitis is characterised by a fulminant, rapidly progressive clinical course; even with aggressive therapy, cases are usually fatal (Lanska, 2002; Sejvar et al., 2005). The likelihood of the development of clinical or subclinical meningitis in patients with severe systemic *B. anthracis* infections is high. In rare cases, anthrax meningitis has been reported without any other associated primary (i.e. cutaneous, gastrointestinal or inhalation) manifestation of anthrax (Lanska, 2002; Sejvar et al., 2005). A review of 82 cases of inhalation anthrax that occurred during 1900--2005 included 70 fatal cases. Among the 70 patients who died, 11 of 61 patients for whom data were available, had signs of meningeal involvement, compared with none of 12 patients who survived; 44 of the 70 patients who died developed meningoencephalitis during the course of their disease, compared with none of the 12 patients who survived. Development of meningoencephalitis during the course of the disease was found to be significantly associated with death ($p = 0.003$) (Holty et al., 2006). Studies in non-human primates have demonstrated meningeal involvement in 33%--77% of experimental inhalation anthrax cases (Friedlander et al., 1993; Gleiser et al., 1963; Fritz et al., 1995; Vasconcelos et al., 2003).

10.5 Anthrax in drug users

A new form of anthrax was observed in drug users in Scotland in December 2009 and similar cases were seen in England during 2010. Drug users may become infected with anthrax when heroin has become contaminated with anthrax spores. This could be a source of infection if injected, smoked or snorted. Patients have not presented with classic anthrax (cutaneous, inhalational or gastrointestinal) but represented a new pattern. The clinical presentation may vary.

The patients that developed intracranial or subarachnoid haemorrhage with anthrax bacilli in their blood died rapidly—i.e. in the late stages of disseminated anthrax. Gastrointestinal symptoms occasionally predominated, probably reflecting disseminated disease. Most have presented as atypical, but severe, soft tissue infections, with significant soft-tissue oedema (one inducing compartment syndrome). Findings differ from classic necrotising fasciitis or classic cutaneous anthrax and can present as variants of cellulitis or abscess. Patients can present with vague prodromal symptoms or excessive bruising at the index injection site,

which may be difficult to identify. Despite appearing very unwell, with tachycardic and peripherally shut down, they maintain an almost normal blood pressure, respiratory function, oxygenation and acid-base, and are lucid. Systemic features might otherwise be non-specific. Haematology and biochemistry are also non-specific; typically the white-cell count, C-reactive protein and lactate are not grossly abnormal. A decline in platelet count may predict clinical deterioration, even if remaining within the normal range. Coagulopathy may develop, with significant bleeding. In cases of severe soft tissue infection, fluid requirements may exceed 10 L per 24 h. Surgical debridement removes the nidus of infection and provides diagnostic material (Gram stain, culture and PCR). Characteristic surgical features include profound capillary bleeding, necrosis of predominantly the superficial rather than deep fat, oedema not fasciolysis and the finding of needle tracks containing necrotic material (Booth, et al., 2010).

11. 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 well-defined territories. Less frequent and certainly more dangerous are introductive events that affect animals living in fixed stalling and 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 need extra feed which will have been 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).

11.1 Differential diagnosis

In cattle, anthrax should be differentiated from the following diseases:

- lightning strike and accidental electrocutions,
- pasteurellosis,
- piroplasmosis ,
- blackleg, malignant oedema and other clostridial diseases,
- food intoxications.

However, we should consider any disease causing sudden death or haemorrhagic septicaemia. 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.

11.2 Laboratory diagnosis

When there is suspicion that an animal has died of anthrax it is important to take precautions to avoid both infection and the shedding of blood that could pollute the surrounding environment. Live animals' blood can be collected from main superficial veins; while with 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 cauterised and we prevent the spilling of blood and ground contamination with spores. 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 the contaminants and encouraging any *B. anthracis* to sporulate. Putrefaction quickly destroys vegetative *B. anthracis* and in this case it is much better to make a swab from nasal turbinates which are well vasculated and therefore should, and do, have plenty of spores, but with minimal tissue are little affected by putrefaction. If 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.

11.3 Microscopic test

A preliminary examination with an unstained fresh blood smear will highlight the presence of stick forms or typical "*bamboo canes*". The organisms are immobile and well capsulated. The slide may be fixed and stained with Gram stain when *B. anthracis* is coloured in violet. Preferably one can use Giemsa which colours 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 K_2CO_3 to 1% has been added (Turnbull, 1998). This with *Bacillus anthracis* 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 who will handle these microscopic preparations (personal observation). Anecdotally there are stories of students getting cutaneous lesions from handling sharp-edged broken blood smear slides that were decades old.

11.4 Cultural test

Bacillus anthracis grows easily on normal agars, whether liquid or solid media. 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 suspect 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 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 materials collected, 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.

11.5 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 has 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.

11.6 Polymerase Chain Reaction (PCR)

To confirm suspicious colonies specific PCR represent the best method to identify *Bacillus anthracis*. To identify virulent *B. anthracis* strains, and for the differentiation of non-virulent strains, the presence of both of the plasmids pXO1 (toxins) and pXO2 (capsule formation) must be confirmed. However, 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).

11.7 Molecular characterisation

The genomic diversity is the result mainly of events in the evolution of the bacterium and the 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 be at low resolution, is the analysis of SNPs (Single Nucleotide Polymorfisms) and identifies point mutations of 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 35,000 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 hard and financially expensive. However, some studies have helped identify 12 canonical SNPs that are the most stable and homoplastic which 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 *B. anthracis* is comparable to 10^{-5} with a high variability depending on the locus (Keim et al., 2004). This technique initially with eight VNTR was able to identify 89 genotypes from 400 isolates from around the world (Keim et al., 2000), while the 15 VNTR assay increased this to 221 genotypes with 1033 isolates. This method has now been increased up to 25 loci (Lista et al., 2006), which

allows an excellent organism discrimination with this high genetic homology. Technically, the VNTR are searched for using capillary sequencers to analyse DNA fragments.

Latterly, high resolution assays were discovered 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 does not 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 four SNR markers that may be discriminated with an advanced method of analysis of DNA fragments (Kenefic et al., 2008).

These briefly 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 their high diversity and homoplasia are able to correctly define genotype, while the latter, searching for any signs of redundancy, are considered suitable for identifying sub-genotypes. All the methods described are best run by specialised laboratories experienced in molecular biology (Keim et al., 2004).

12. Anthrax vaccines and their mechanism of protection

Toxin formation is known to occur when PA binds to receptors on cells (Little et al., 2004; Bradley et al., 2001), undergoes proteolysis which exposes a binding site for LF or EF and forms heptamers (Milne et al., 1994). The shared cell-binding component, PA, when combined with LF, forms a lethal toxin, which kills laboratory animals (Beall & Dallford, 1966; Stanley & Smith, 1961) and is cytotoxic to certain macrophage cell lines (Friedlander, 1986). When combined with EF, on the other hand, PA forms an oedema toxin, which causes oedema and inhibits neutrophil functions (O'Brien et al., 1985) due to the calmodulin-dependent adenylate cyclase activity of EF. Clearly, then, blocking PA leads to the neutralisation of the toxic activity of anthrax. Indeed, protection of certain animal models (guinea pig, rabbit, non-human primate) against infection with *B. anthracis* can be achieved by inoculation with a variety of vaccine preparations that contain PA as their main immunogen (Ivins et al., 1990; Ivins et al., 1992; Ivins et al., 1998). Moreover, a strong correlation has been found between the level of PA-specific toxin-neutralising antibodies (TNA) and protection.

Toxin neutralisation is probably not the only antibody-mediated mechanism of protection. The kinetics of PA production during *B. anthracis* growth and the role of anti-PA antibody in host immunity are not clearly defined, however. Recently, anti-PA antibodies (Abs) have also been shown to exhibit anti-spore activities. Rabbit anti-rPA polyclonal Abs (pAbs) were shown to enhance the phagocytosis and subsequent killing of spores by macrophages, and to partially inhibit spore germination in vitro. Further, PA was found to be associated with spores and to induce anti-PA Abs which retard germination in vitro, and enhance the phagocytic and sporicidal activities of macrophages (Cote et al., 2005; Welkos et al., 2001; Stepanov et al., 1996; Welkos et al., 2002).

An important aspect of the protective ability of the immune system is the persistence of PA-specific IgG memory B cells allowing animals to remain resistant to infection even after their serum Ab response has waned. In a study on mice, for example, half of the animals immunised with CpG-adjuvanted AVA (Synthetic Oligodeoxynucleotides containing Unmethylated CpG motifs to AVA) with anti-PA titers 10-fold below the protective baseline, survived a 100 LD_{50} Sterne strain spore challenge. This contrasted with only 1/35 mice with the same Ab titer that had been immunised with AVA alone. These findings suggest that an important goal of anthrax vaccine development should be that of attaining a vaccine able to generate a durable pool of high-affinity memory B cells (Tross & Klinman, 2008; Ivins et al., 1994).

Another important aspect of immunity is with regard to T cells which may play a role beyond simply enhancing adaptive humoral response. Immunisation with formaldehyde-inactivated *B. anthracis* spores resulted in the generation of CD4 T lymphocytes, which responded in an MHC-restricted manner by producing interferon γ (IFNγ) (Glomski et al., 2007). This suggested that the production of IFNγ leads to the activation of phagocytes and consequently increases sporicidal and bactericidal activity. IFN was shown to protect up to 60% of mice against lethal inhalational anthrax (Walberg et al., 2008). Finally, nasal (i.n.) immunisation of deeply anesthetised rabbits with rPA+IL-1α consistently induced rPA-specific serum IgG ELISA titers that were not significantly different than those induced by intramuscular (IM) immunisation with rPA+alum, although lethal toxin-neutralising titers induced by nasal immunisation were lower than those induced by IM immunisation (Gwinn et al., 2010).

12.1 First generation of anthrax vaccine for human use

The observation that the injection of sterilised oedema fluid from anthrax lesions in laboratory animals protected against challenge with a fully virulent strain, suggested that the acellular vaccine can protect against anthrax. Investigations followed on the protective role of artificially cultivated *B. anthracis* filtrates as vaccines for human use. The first US product was developed in 1954. It was a cell-free filtrate from an aerobic culture of the Vollum strain of *Bacillus anthracis*, precipitated with aluminium potassium sulphate. In the 1960s, the strain used was changed from Vollum to V770-NP1–R, a toxigenic, non-capsulated and non-proteolytic mutant (Puzzis et al., 1963) and the microaerophilic culture method adopted. A significant increase in the stability and immunogenicity of the vaccine was obtained as a result. This vaccine, named Anthrax Vaccine Adsorbed (AVA), was licensed by the NIH in 1970 and reapproved by the FDA in 1985. In December 2008, the FDA approved a biologics licence application supplement for AVA, submitted by Emergent BioSolutions. The current licensed schedule consists of five of 0,5 ml intramuscular injections (at zero and four weeks, and at six, 12 and 18 months) followed by yearly 0,5-ml booster doses. Intramuscular injection causes fewer local reactions, but it entails a reduction in anti-PA antibody response from week eight to six months after vaccination, during which protection may also be reduced (Wright et al., 2009).

The Anthrax Vaccine Precipitated (AVP) was licensed in Great Britain in 1979. It was developed by the Centre for Applied Microbiology and Research at Porton Down, Salisbury, using an avirulent toxigenic, non-capsulated 34F2 strain of *B. anthracis* originally isolated by Sterne in 1937. It contains PA, LF and EF. The main indication for using the vaccine is risk of infection by inhalation of *B. anthracis* spores.

The AVP vaccine is administered in a three-dose primary regimen three weeks apart, followed by the fourth dose after six months and annual booster doses. The main active component of the vaccine is a sterile filtrate of alum-precipitated *B. anthracis* antigens in solution for injection. Other ingredients are aluminium potassium sulphate, sodium chloride and purified water. The preservative is thimerosal (0.005%). Immunisation by the vaccine induces production of IgG antibodies, which guarantees good immunogenicity. No serious side effects have been reported. Reactions are uncommon, but occasionally a mild rash or swelling at the site of injection, or even at the site of an earlier injection, may occur and last for a couple of days. More rarely, swollen glands, mild fever, flu-like symptoms, a rash, itching or other allergic reactions may occur (Baillie, 2009; Friedlander & Little, 2009; Splino et al., 2005).

Compared to AVA, the British AVP contains lower levels of PA and higher concentrations of additional *B. anthracis* antigens, such as LF and EF, and certain bacillus surface proteins (Turnbull, 1991; Baillie et al., 2003). These differences, owing to the strain used and/or to vaccine preparation techniques, may be the cause of the slightly enhanced protection conferred by AVP (Baillie et al., 2004) and of the increased transient reactogenicity seen in comparison to AVA (Turnbull, 2000). First-generation vaccines are, thus, relatively safe and efficacious, but they do present a number of important limitations, making them less than ideal for urgent mass vaccinations or for use in non-industrialised or remote regions.

12.2 Second generation of anthrax vaccine for human use

12.2.1 PA vaccines

Several high-level PA expression systems have been developed based on a variety of microbial and eukaryotic organisms such as attenuated strains of *B. anthracis*, *B.subtilis*, *B. brevis*, *Baculovirus*, *Escherichia coli* (Baillie, 2006).

rPA102 (formerly produced by Vaxgen Inc., South San Francisco, California, later acquired by Emergent BioSolutions, Maryland) is a purified protein obtained from culture supernatant of *B. anthracis* ΔSterne-1, an asporogenic, avirulent, non-toxigenic strain, which contains a recombinant plasmid encoding PA. PA is adsorbed in aluminium hydroxide adjuvant with a final aluminium concentration of approximately 82.5 µg per dose. This vaccine protected rabbits and non-human primates from inhalational challenge and was found to be safe and immunogenic in a randomised trial performed on healthy volunteers (Gorse et al., 2006). SparVax, an rPA vaccine obtained from *E. coli* (Baillie, 2009) and manufactured by Pharmathene in the US, is undergoing US National Institute of Health-sponsored human safety and immunogenicity trials. SparVax was developed by researchers at the Defence Science and Technology Laboratory, Porton Down, Wiltshire UK.

In preclinical studies, SparVax has demonstrated the capability to protect rabbits and non-human primates against a lethal aerosol spore challenge of the anthrax Ames strain.

A recently published report described a phase I clinical trial testing the safety and immunogenicity of an anthrax vaccine using *Escherichia coli*-derived, *B. anthracis* rPA. Sixty seven healthy adults received two injections, four weeks apart, of either rPA in increasing doses (5, 25, 50, 100 µg), formulated with or without 704 µg/ml Alhydrogel adjuvant, or buffered saline placebo. Participants were followed for one year. No serious adverse events were recorded. The most robust humoral immune responses were observed in subjects

receiving 50 μg of rPA formulated with Alhydrogel, while the strongest cellular response was observed in the group receiving 25 μg Alhydrogel-formulated rPA. The vaccine was safe, well tolerated and stimulated a robust humoral and cellular response after two doses (Brown et al., 2010).

12.3 Third generation of anthrax vaccine for human use

12.3.1 Epitope–specific vaccines

The efficacy of PA domain was demonstrated in mice: all animals immunised with PA proteins containing domain 4 were fully protected against anthrax spore challenge while a decrease in protection was seen in mice immunised with a mutated strain of *B. anthracis* that expressed PA without domain 4 (Flick-Smith et al., 2002; Brossier et al., 2000).

12.3.2 Oral vaccines

Aloni-Grinstein et al. showed the efficacy of an attenuated non-toxigenic non-capsulated *B. anthracis* spore vaccine as an oral vaccine in guinea pigs (Aloni – Grinstein et al., 2005).

Another orally delivered vaccine for human use is that derived from *Salmonella enterica* serovar *Typhimurium.* Vaccines based on either full-length PA, PA domains 1 and 4 or PA domain 4 were tested on A/J mice. The study compared oral vaccines with rPA vaccines showing, for the first time, the efficacy of an oral *S. enterica*-based vaccine against an aerosolised *B. anthracis* challenge (Stokes et al., 2007; Baillie et al., 2008).

Orally administered *Lactobacillus gasseri* engineered to express the PA-DCpep fusion proteins was proven effective against anthrax Sterne challenge. This vaccine showed efficacious adjuvanticity and a safe delivery to mucosal immune cells, including dendritic cells. Both mucosal and systemic immune responses were elicited, resulting in complete animal survival (Mohamadzadeh et al., 2010).

12.3.3 Nasal vaccines

Rapid protective immunity has been achieved in mice through a combination of a nasal prime with a *S. Typhi* vaccine strain expressing PA, followed by a parenteral rPA boost. The same immunising strategy using a *S. enterica* serovar *Typhi*-derived PA83 fused with the export protein ClyA (ClyA-PA83) was also tested in rhesus and cynomolgus macaques. Monkeys developed high levels of serum TNA. Having been successful in non-human primates, this anthrax vaccine strategy based on heterologous mucosal immunisation followed by a parenteral vaccine booster is considered very interesting for human application (Mikszta et al., 2005).

12.3.4 DNA vaccines

A human serotype 5 adenovirus (ad5) expressing PA (AdsechPA) was tested and compared with the new US military rPA/Alhydrogel vaccine in a mouse model. AdsechPA afforded approximately 2.7-fold more protection than the rPA vaccine against *B. anthracis* lethal toxin challenge four weeks after a single intramuscular administration, suggesting the potential of this vaccine to protect the civilian population against *B. anthracis* in response to a bioterrorism

attack (Tan et al., 2003). Chimeric virus-like particles (VLPs), which are very effective at eliciting humoral as well as cellular immunity, were also tested. VLPs complexed with PA elicited a powerful TNA response that protected rats from anthrax lethal toxin challenge after a single dose without adjuvant. This highly effective, dually-acting reagent can be used both for protection against anthrax and as post-infection treatment (Manayani et al., 2007).

12.4 Vaccine for veterinary use

The history and theory of anthrax vaccines for veterinary use are closely linked to the first developments of modern vaccinology science. Louis Pasteur, a pioneer in this field, developed the first anthrax vaccine in 1881 (Shlyakhy et al., 1996). His method was widely used for livestock immunisation until the 1930s. Pasteur's schedule consisted of a first inoculation of *B. anthracis* cells from cultures incubated at 42°- 43°C for 15-20 days (Pasteur type I) followed by an inoculation, after 14 days, of less attenuated *B. anthracis* cells from cultures incubated at 42°- 43°C for 10-12 days (Pasteur type II) (Turnbull, 1991).

The live attenuated vaccines for veterinary use can be divided into three main categories: Pasteur vaccines, Carbozoo vaccines and Sterne vaccines. The division is not merely historical, but based on different attenuation mechanisms (Hambleton et al., 1984) . The Pasteur method of attenuation results in the loss of the pXO1 plasmid that encodes the major virulence factors (PA, LF, EF), thus producing a non-toxigenic and capsulated (pXO1-,pXO2+) vaccine. The Sterne type is a *B. anthracis* strain lacking the pXO2 plasmid encoding the capsule. It is, therefore, toxigenic and non-capsulated (pXO1+,pXO2-), resulting in a non-virulent stable phenotype which still conserves the main antigen, anthrax toxins. The Carbozoo attenuation mechanism is still unknown, but studies on Carbosap demonstrated the presence of both plasmids (pXO1+ pXO2+) placing this strain in the category of toxigenic and capsulated, and suggesting different mechanisms of attenuation (Fasanella et al., 2001). At present, most veterinary vaccines are live attenuated vaccines, produced worldwide according to the requirements for anthrax spore vaccine (live- for veterinary use), the requirements for biological substances No. 13 (WHO, 1967), the manual for the production of anthrax and blackleg vaccines (FAO, 1991), the manual of diagnostic tests and vaccines for terrestrial animals (OIE, 2008) and the updated European Pharmacopoeia. The Sterne 34F2 strain is used worldwide, with the exception of Russia, China and Romania, where other, analogous toxigenic and non-capsulated strains are used. The formulation consists of about 10^7 spores suspended either in glycerin with saponin or in physiological solution with saponin. The effectiveness of this vaccine soon emerged, with a sharp reduction in outbreaks observed in South Africa during the period 1925-1941. Moreover, epidemiological data suggest that, in the past 50 years, vaccination has drastically reduced anthrax in industrialised countries where it is now considered rare.

New animal vaccines are sorely needed. First studies reporting the use of recombinant or edible vaccines for veterinary use have been conducted. These proved the efficacy of two experimental vaccines against *B. anthracis* for veterinary use: an rPA mutant vaccine and a trivalent vaccine (TV) composed of rPA, an inactive LF mutant (mLF-Y728A; E735A) and an inactive EF mutant (mEF-K346R), both emulsified with mineral oils. Although this was only a preliminary study on a rabbit model, the possibility of administering these vaccines with antibiotics to halt incubating infections or during an anthrax epidemic was underlined (Fasanella et al., 2008).

Preliminary attempts to generate transgenic PA-producing plants successfully explored the possibility of creating a safe and protective vaccine. The use of an edible vaccine would be useful for the vaccination of herbivores - both domesticated and feral. Anthrax control programmes would be improved above all in non-industrialised countries, where syringes and needles are normally in short supply.

For example, in the search for an alternative, less expensive method to produce PA, a transgenic tobacco chloroplast was developed, that expressed the 83 kDa immunogenic *B. anthracis* PA. Crude plant extracts contained up to 2.5 mg full length PA/g of fresh leaf tissue and this showed exceptional stability for several months in stored leaves or crude extracts . The recently demonstrated efficacy of plant-expressed domain 4 of *B. anthracis* PA opens new horizons for the mass vaccination of animals in areas where the risk of anthrax is high (Watson et al., 2004; Brodzik et al., 2005; Gorantala et al., 2011).

13. Conclusions

Anthrax is an infectious disease which is still widespread in many areas of the planet and its presence is recorded mainly in poor or developing countries where the lack of an efficient health system able to prevent or counteract health emergencies favours the spread of infections, which often tend to result in an epidemic form. The source of anthrax infection is animals and the controlling of the disease in animals reduces the risk of human infection. The vaccine is still the most effective means of control, but mass vaccinations are not always possible in underdeveloped areas, where in addition to the lack of infrastructure such as roads or passable roads, an information system on the real animal population to submit to the vaccine treatment is often absent. Programmes to combat zoonoses, and anthrax in particular, need to have a fruitful collaboration between health authorities and farmers who need to be active players in the programme and not passive spectators. The process of training and information of those active in agriculture on the real dangers of the infection is fundamental, envisaging the adoption of restrictive measures in the case of outbreaks and not penalising the fragile economy of the livestock sector. The recent epidemic in Bangladesh owes its spread to the fact that farmers, fearing economic losses linked to the deaths of their animals, slaughtered them during the illness or even in that pre-agonising phase to sell the meat at a reduced price to limit losses.

With regard to developed countries, except for the anthrax threat represented by its use as a bacteriological weapon or potential bioterrorist attacks and episodes in drug users, anthrax is a sporadic disease characterised by few outbreaks that tend to occur where infected animals were buried in the past or where collected waste from the leather industries was placed. More frequently are reported outbreaks that develop consequent to the introduction of contaminated feed.

In wild areas like natural parks or natural reservations, human control is not always efficacious and often the carcasses of dead animals are left undisturbed by scavengers. Since the carnivores are less susceptible to the disease compared to herbivores, they can ingest larger quantities of infected viscera and meat, but the vegetative cells do not survive passage through their acid stomachs; but if they have been eating older carcasses with spores they may spread spores in their faeces (Turnbull et al., 1989). In wild 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. These events permit generation of a relevant amount of spores that spread in the environment. It seems that *B. anthracis* has found in wild areas its natural habitat that permits the completion of its cycle and the production of a sufficient amount of spores ensuring its survival.

The area located between agricultural and wild areas where generally human activity is limited to the exploitation of pastures represents the contact point between the wild and the agricultural world, the habitat where domestic and wild animals divide the same space and where the ecology systems tend to influence each other. The proximity to the sources of production of anthrax spores, that are located in the wild area, guarantees the standard level of contamination of soil, favouring the realisation of the events that cause the disease in the domestic animals that pasture on this area, in conclusion, the area where anthrax crosses the border of its habitat and shows its presence. Animals from areas free of anthrax placed in areas at risk would be much more receptive to the disease. The project for the reintroduction of deer in some nature reserves of Basilicata (South Italy) is facing major obstacles just because of the receptivity of this particular animal species to anthrax infection (Fasanella et al, 2007). We do not know if this is a form of sensibility related to animal species or related to a lack of natural antibodies, but it is certain they are subjects who come from ecosystems in which anthrax is not present. In nature there are no behaviours which are an end in themselves and every living being has evolved its own strategies, not only in terms of preservation of their species, but also in that of its ecosystem. So why not hypothesise that *Bacillus anthracis* returned to its protective role of the delicate balance of its ecosystem, protecting the animal species that are an integral part of that particular area from a possible risk of extinction, due to infectious diseases introduced by unknown animals from different environments.

In developed countries, where the disease is sporadic, anthrax can cause serious health problems when it develops outbreaks in areas considered free of the disease, because the real risk is that health authorities can intervene in a misdiagnosis and not take the necessary precautionary measures. In conclusion, we must begin to consider anthrax as a neglected disease and undertake all activities that tend to reduce the risk for humans related to the underestimation of the disease. It is necessary to continue in the research activity on new and more sensitive and rapid diagnostic tests, on the development of more effective vaccines for human and veterinary use, and also on the improvement of the information and training of health personnel responsible for the control of zoonoses.

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15. References

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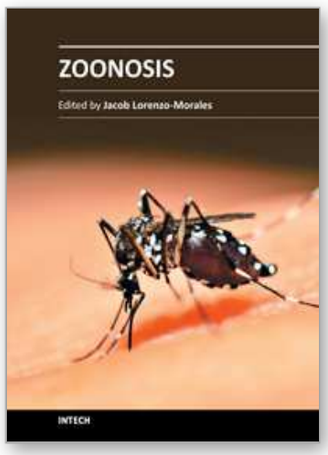

Zoonosis

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Zoonotic diseases are mainly caused by bacterial, viral or parasitic agents although "unconventional agents" such as prions could also be involved in causing zoonotic diseases. Many of the zoonotic diseases are a public health concern but also affect the production of food of animal origin thus they could cause problems in international trade of animal-origin goods. A major factor contributing to the emergence of new zoonotic pathogens in human populations is increased contact between humans and animals. This book provides an insight on zoonosis and both authors and the editor hope that the work compiled in it would help to raise awareness and interest in this field. It should also help researchers, clinicians and other readers in their research and clinical usage.

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