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Bacterial Spores and its Relatives as Agents of Mass Destruction

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Abstract

The term bioterrorism has acquired full force for the planetary consciousness from the very beginning of the new century. Indeed, from the events that occurred during October and November of 2001 with the intentional contamination with spores of pathogenic *Bacillus anthracis*, of letters distributed by the US public postal service and the terrorist attacks in the last months of 2015 in Egypt, France, Mali, Afghanistan, Turkey, USA and other countries have warned again about the reality of the bioterrorist threat and its immeasurable cultural and undesirable economic and political consequences. In this review, we summarize the main structural characteristics that make the spores of Bacilli and Clostridia as the ideal agents for use in bioterrorism. In addition, we discuss the properties of non-sporulating *Coxiella burnetii*, the causative agent of Q fever, because of its peculiar resistance, high infectivity and environmental persistence that resembles true spores.

Keywords: Spores; Anthrax; Botulism; Gas gangrene; Diarrhoea; Q fever; Prevention response to bioterrorism

Introduction

Bioterrorism in the world today

Biological weapons, as opposed to nuclear weapons, are easier to produce and require far less money investment and human resources [1-8]. It is believed, that if an attacker (a country, a sect or an individual) wants to harm another (people of a country), the economic cost to affect the enemy territory using a nuclear weapon will be considerably much higher than using a biological weapon [8]. Therefore, due to its low cost, ease of production and fearful danger it is that biological weapons pose a real threat to modern societies [2,4,6]. Unlike a biological warfare (between countries) or a biocrimen (against one person) in a bioterrorist attack the general civilian population is the target. In this sense, an act of bioterrorism can be defined as the intentional use of live biological agents (mainly bacteria and viruses) or their derivatives (mainly toxins) to cause panic, illness and /or death in the population. Because of the relative ease of developing biological weapons not only states or nations are able to sponsor bioterrorist attacks but also sects, fundamentalists and even individuals, i.e. a lone wolf terrorist, who wish to do so [1,4]. Today all mankind is threatened of a possible attack with a weapon of mass destruction. Even in cases of false alarm, the economic costs to the threatened country to originate prevention and implementation of response plans are repeatedly millionaires in terms of national budgets [9-14], not to mention the psychological damage on the civilian population that hurts the society narcissism in the depths of its being [2-4,9-14]. If what is sought is the biggest physical and / or psychological damage with the lowest economic cost then: what should be the ideal characteristics of a biological agent as a weapon of mass destruction? Well, it should comply with as much of the following characteristics: be cheap, easy to hide and to produce in large quantities, should have no smell, taste or characteristic colour that reveal its presence before unleashing its toxic and/or lethal effect, should be able to be aerosolized, must survive as long as possible to its exposure to sunlight, should be as resistant as possible to drying and heat, it should be capable of producing massive death or serious illness (i.e. have a high infectivity rate even in a low dose), must be contagious from person to person, and there should not be available (at least in a timely manner) medical treatment or prophylaxis once warned the attack [2-4,9]. Among all these features perhaps aerosolization deserves a further comment due to its importance in case of a bioterrorist attack [1,2]. This term refers to the particle size that the microbial agent should have (1 to 10 micrometres in diameter) to effectively spread through the air. For comparison, a particle of 1 mm of diameter is able to travel through the air along a distance of 1 meter before falling, whereas a particle of 0.5 microns of diameter travels through the air over 1,000 (thousand) kilometres before "settle" on a surface or be unnoticed inhaled (breathed) by a person, reaching the deepest parts of the lungs (the alveoli) from where the infectious agent spreads to cause disease and / or death [15-17]. Precisely, in the microbial world, bacteria and viruses are the infectious agents that perfectly meet the sized requirement to be compacted into fine powders feasible to spread through the air. The infective powder will access the human alveoli and will start the disease that in all cases will go unnoticed as a simple cold or flu during the first days until to show up as it really is (Anthrax, Botulism, Gas gangrene, Q fever, etc.). Unfortunately, by that time, the medical treatment would, in most cases go, fruitless. When cells of certain Gram-positive bacteria encounter environmental stresses such as nutrient starvation, they form a dormant structure termed a spore [18,19]. Bacterial spores can survive in this dormant state for many years. Faced with the challenge of surviving prolonged periods of dormancy, spores have evolved many mechanisms to protect themselves from damage, which also serve to protect them from modern disinfection/sterilization procedures [20-23] (Figure 1).

Microbial agents of mass destruction

Biological agents that could or have been used as biological weapons of destruction or mass panic can be divided into two types:

- Live microorganisms (bacteria, fungi and viruses).

- Poisons or toxins derived from microorganisms (mainly bacteria and fungi), plants, snakes, etc.

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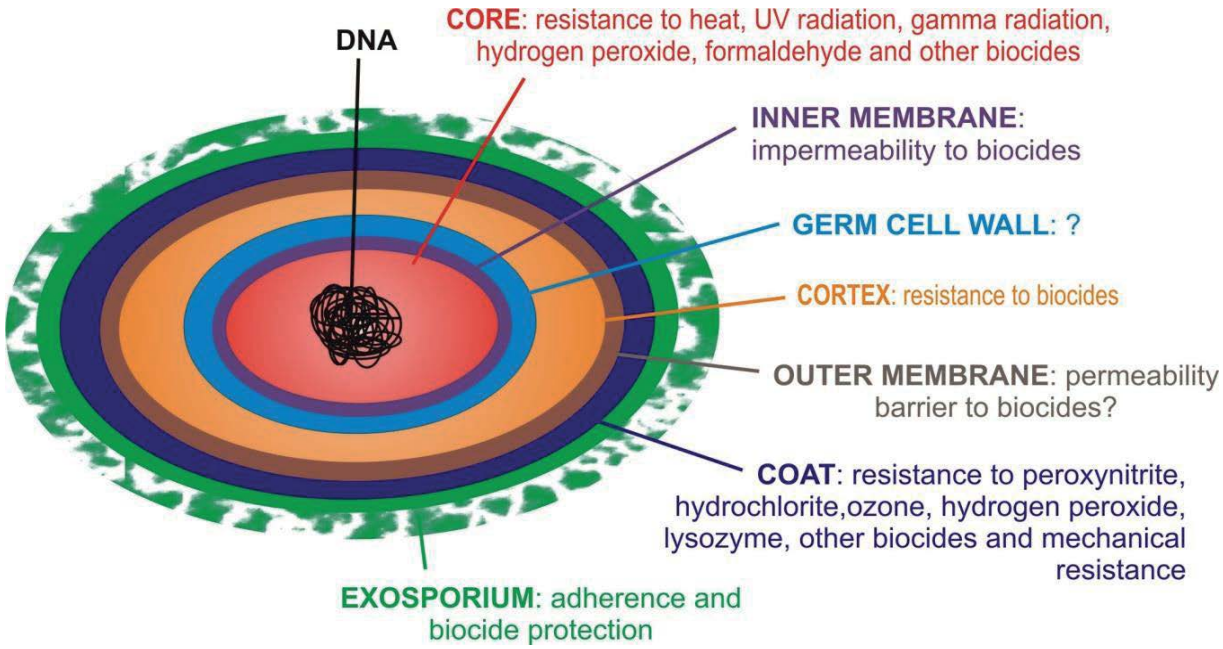

Figure 1: Cartoon of a typical bacterial spore. The different structural components of the spores of *Bacilli* and *Clostridia* and their roles in resistance to biocides and chemical/physical treatments are indicated here and explained in the text.

Evidently, microbes play a central role as agents of bioterrorist utilization. Perhaps the most useful classification is given by the Centre for Infectious Disease Control (CDC) which ranks microorganisms in three Categories or Classes (A to C) according to the suitability for their use in bioterrorism acts, on how easily can be spread and the severity of illness or death they cause (www.emergency.cdc.gov/bioterrorism) (Table 1). Following these criteria, category A agents are considered the highest risk and category C agents are those considered emerging threats for disease (Table 1).

In Class A, there are the most dangerous microbes, i.e. those who meet the highest number of "desirable" properties of a bioterrorist agent (bacteria or virus) mentioned before and listed in (Table 1). In category B are included those agents who have been assigned a low priority and those in category C are of relative priority, including hypothetical or emerging pathogens. Examples of Class A microorganisms and diseases caused by them are Ebola virus, *Bacillus anthracis* (anthrax), *Yersinia pestis* (plague), *Clostridium botulinum* (botulism toxin), *Francisella tularensis* (tularemia), and *Variola virus* (smallpox). Examples of Class B microorganisms are *Burkholderia* sp. (melioidosis, glanders), *Brucella* sp. (brucellosis or undulant fever), *Coxiella burnetii* (Q fever), *Chlamydia psittaci* (psittacosis), *Clostrifium perfringens* (epsilon toxin) and Hepatitis A virus.

In this review, we want to emphasize those microorganisms capable of forming the strongest living structure that the human knows: the spore cell (Figure 1) [18-20]. Bacterial spores could represent bioterrorist agents of spectacular concern in case of used by the wrong hands. In this sense, the Class A microorganisms *C. botulinum* and *B. anthracis* and the Class B microorganism *C. perfringens* belong to the select group of spore-forming bacteria indicated by the CDC. It is important to consider that in the case of *C. botulinum* (Class A) and *C. perfringens* (Class B), the CDC only takes into consideration the bioterrorist use (in food and water) of the botulism toxin and epsilon toxin, produced by *C. botulinum* and *C. perfringens*, respectively, and not the bioterrorist utilization of aerosols containing spores of these bacteria as it is the case for *B. anthracis* [24-26]. Therefore, we want to analyse here the potential use of aerosolized *C. botulinum* and *C. perfringens* spores in bioterrorism.

In addition, we also consider the potential use of the aerosolized pathogens *C. difficile* and *Coxiella burnetii*. *C. dificcile* is responsible for the production of deadly diarrhoea in humans but is not considered by the CDC as a biological agent of potential bioterrorist use. However, recently emerged strains of this spore-forming bacterium, considered as "superbug", are multi resistant to antibiotics and more virulent and therefore we hypothesize that *C. difficile* spores might be successfully used in a bioterrorist attack [27,28]. By other side, the class B agent *Coxiella burnetii* (Table 1) is the etiological cause of Q fever in animals and humans [29] but it does not sporulate. However, although *C. burnetii* does not produce real spores we include this pathogen in the present review because produces a unique cell (small cell variant, SCV) of high robustness, persistence and infectivity that resemble a bacterial spore [30].

The bacterial spore: structural features for its high resistance and virulence

Some spore-forming bacteria, belonging to the *Clostridium* and *Bacillus* genera, are associated with diseases [20]. Examples of these illnesses are tetanus (*Clostridium tetani*), botulism (*Clostridium botulinum*), gas gangrene (*Clostridium perfringens*), CDI (*Clostridium difficile* infections) and anthrax (*Bacillus anthracis*). Many of these diseases are not frequent in developed countries because the use of appropriate hygienic practices, vaccines and right antibiotics. Nevertheless, highly virulent variants of these pathogens have emerged, because of the overuse/misuse of antibiotics, as well as the genetic modification of spore-forming strains [31-35]. In addition to this

]Category A (natural or engineered organisms or toxins that represent the highest risk to the public and national security)	Category B (moderately highest priority)	Category C (emerging pathogens)
◊ They can be easily spread or transmitted from person to person, ◊ They result in high death rates and have the potential for major public health impact ◊ They might cause public panic and social disruption ◊ They require special action for public health preparedness	◊ They are moderately easy to spread ◊ They result in moderate illness rates and low death rates ◊ They require specific enhancements of CDC's laboratory capacity and enhanced disease monitoring	◊ They are easily available ◊ They are easily produced and spread ◊ They have potentiality for high morbidity and mortality rates

Table 1: Main characteristics used by CDC to classify bacterial agents in function of the probability of their utilization and the potentially danger after a bioterrorist attack.

clinical notoriety, these two bacterial genera (*Bacillus* and *Clostridium*) have also been very successful to survive in the environment, due at least in part to their ability to form spores (Figures 1 and 2). Spores and spore-forming bacterial species have been found in nearly every habitat on earth, including as part of the normal flora of animals and human beings. Due to their inherent resistance to decontamination as well as unique biology, bacterial spores pose a particular challenge to infection control, food storage and safety and traditional clinical interventions [18-21] (Figure 1). Understanding the role that spores play in the environment as well as in disease, will be an important step towards mitigating the threat posed by these bacteria [20-23].

The differentiation of actively growing (vegetative) *Bacillus* and *Clostridium* organisms into spores is a multistep process that occurs in response to environmental and metabolic clues (Figure 2A) [18,19]. The sporulation process is a carefully orchestrated cascade of events at both the transcriptional and post-translational levels involving several sigma factors of the RNA polymerase, transcription factors, proteases, phosphatases and quorum sensing molecules [18,19]. Upon completion of the process, the spore is released into the extracellular milieu by autolysis of the mother cell (Figure 2A). The entire developmental program takes, in the laboratory under optimal conditions, approximately 8h [18]. Dormant spores can remain viable for extremely long periods of time in the environment, and are highly resistant to adverse conditions [15,21-23]. The concentric series of structures that formed the spore are responsible for its resistance and longevity (Figure 1).

Spores have evolved to germinate (or reactivate) inside a host (in response to the presence of nutrients or germinants) causing disease because of toxin production (Figure 2B) [20].

Bacterial spores are likely the most resistant and durable form of life on Earth. The spore structure and chemical composition play major roles in spore resistance. Spores are resistant to a variety of antimicrobial and biocide compounds, extremes of temperature and pH, UV and ionizing radiation, and can survive for extreme lengths of time without water or nutrients [22,23]. Furthermore, the spore's core is dehydrated with a concomitant increase in Ca^{2+} and dipicolinic acid (DPA) concentrations which jointly protect against heat stress (Figure 1) [22,23]. In addition, spore's DNA is enveloped by a specialized class of proteins called small acid-soluble proteins (SASPs), which protect the DNA from UV and gamma radiations during dormancy [22,24-37]. Since spores are metabolically dormant, bacteria have the advantage to pass unharmed and unnoticed through diverse environments before germination (Figure 2A). Starting from the outside of the spore, the spore layers include the exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane and central core (Figure 1) [18,22,23].

Structural analyses of spores have revealed that they possess a flexible balloon-like structure (Figure 1). The exosporium is the outermost structure of many bacterial spores, in particular those of the *B. cereus* group, which also includes *B. anthracis* and *B. thuringiensis*, but is also found in some other Bacilli and Clostridia, including the pathogenic *C. difficile* and *C. botulinum* [20-23]. The exosporium, which has been shown to protect the spores, facilitates the adherence to biotic and abiotic surfaces, allowing the spores to spread between infected individuals and between contaminated and uncontaminated surfaces [20,23,38-40] (Figure 1). Therefore, adherence of spores to host-cells has been suggested to be a critical virulence factor. Adherence plays a key role in the spore infectious potential. This is of particular concern in the hospital setting, where walls, floors, appliances and other surfaces, as well as healthcare workers themselves, can become a reservoir of infectious particles [40]. Spores of *Bacillus anthracis* adhere to cells of the airway and intestinal epithelia, a property that potentiate the virulence of this pathogen [41,42]. A collagen-like protein of the *B. anthracis* exosporium (BclA) has been implicated in the binding to the Mac-1 cell surface receptor of macrophages, inducing its own phagocytosis by those host cells [43]. In addition, the immune-inhibitor A protein (InhA) of *Bacillus cereus*, a metalloprotease found in the exosporium, has also been found to be involved in escape from the macrophage endosome after spore internalization [39-44].

The spore coat is a complex structure composed of several layers and is made up mainly of proteins, most of which are spore-specific gene products. The coat is important in spore resistance to exogenous lytic enzymes that can degrade the spore cortex and to predation by protozoa and to some chemicals, especially oxidizing agents such as hydrogen peroxide, ozone, peroxynitrite, chlorine dioxide and hypochlorite, all of which kill spores more rapidly when the coat layer is absent, but has little or no role in spore resistance to heat, radiation and some other chemicals [22,23,45].

The precise function of the outer membrane that lies under the spore coats is not clear, although this membrane is an essential structure in spore formation [18,19,22]. The cortex is composed of peptidoglycan (PG) with a structure similar to that of vegetative PG but with several spore-specific modifications [18,19,22,46]. The cortex is essential for formation of a dormant spore and for the reduction of the water content of the spore core. The inner spore membrane is a strong permeability barrier that plays a major role in spore resistance to many chemicals, in particular those that could damage the spore DNA [21-23,36,37].

Finally, the core contains most spore enzymes as well as DNA, ribosomes and tRNAs. The low core water content is likely the major factor in the spore's enzymatic dormancy, and is the most important factor determining the spore's resistance to wet heat. As mentioned before, a core small molecule important in spore resistance is DPA [36,37]. This molecule comprises 5–15% of the dry weight of spores of both *Bacillus* and *Clostridium* species and is located only in the core, where it is most likely chelated with divalent cations, largely Ca^{2+}. DPA plays a significant role in the UV photochemistry of spore DNA

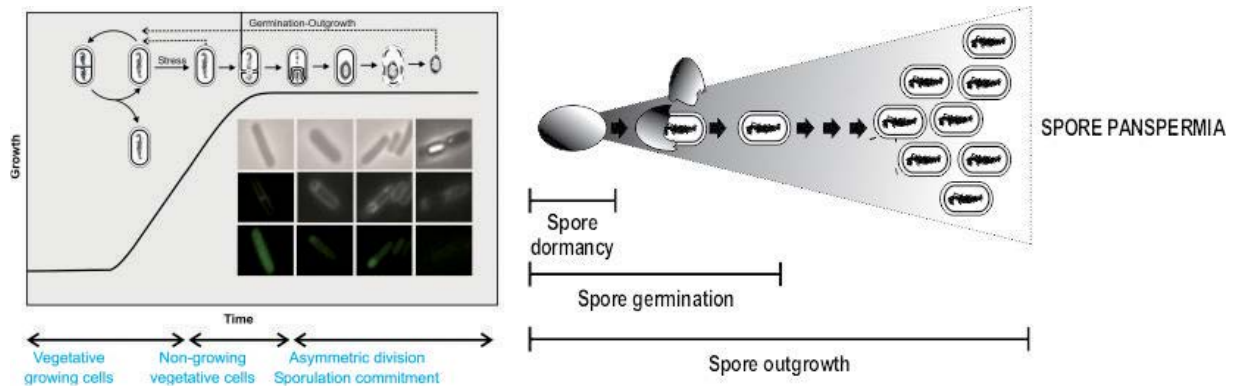

Figure 2: The sporulation and germination cycle of *Bacilli* and *Clostridia*. (A) When a vegetative cell of *Clostridium* or *Bacillus* reaches the end of the exponential phase of growth start a developmental process that ends with the formation of a robust and long lasting spore. (B) When the spore finds a propitious environment and specific germinants start a process of spore germination and outgrowth that with the emergence of the vegetative cell that was encased into the spore shield. The emerged vegetative cell is able to start new rounds of vegetative division to colonize biotic and abiotic surfaces (spore panspermia). See text for further details.

[36,37]. Another type of core molecules that play an important role in spore resistance is the SASPs. The binding of these proteins alters DNA's structure and properties dramatically, and are significant factors in spore resistance to heat and many chemicals, and a major factor in spore resistance to UV radiation [21-23,36,37]. In addition to preventing DNA damage, the spore has another mechanism to minimize damage: it has a rapid and efficient DNA repairing mechanism of any DNA damage that could happen during spore dormancy that is activated during spore outgrowth before takes place the first division cycle of the emerged cell (Figure 2B) [22,37]. It has been suggested that superoxide dismutase (SOD), an enzyme associated with the exosporium or spore coat of *B. cereus* and *B. anthracis,* is involved in the formation of the spore coat, may also serve to detoxify potentially damaging chemicals at the spore surface [23,47]. Other role also exhibited by the activity of SOD enzymes is to evade host responses. Spores of *B. anthracis* contain several superoxide dismutases, which provide resistance to the oxidative burst produced by macrophages after the phagocytosis of *B. anthracis* [23,47]. In addition, a nitric-oxide synthase activity in *B. anthracis* spores also provides protection from macrophage-mediated killing, complicating medical treatment [41,42]. Spore resistance can also play a more direct role in disease development by allowing spores to survive attack by the host's phagocytic cells. For *Clostridium difficile,* the causative agent of antibiotic-associated diarrhoea, transmission of spores between infected patients and healthy persons or surfaces is common [48,49]. Ingested spores also survive the degradative enzymes and low pH of the stomach, facilitating their passage into the lower gastrointestinal tract where they can germinate and initiate infection [48,49].

One step required for pathogenic spores to initiate an infection (once inside the host) is the transformation of spores into vegetative cells by a process called germination (Figure 2B) [50,51]. Therefore, spores must activate germination only when conditions are favourable for survival of the nascent vegetative cells. To this end, spores have evolved sensitive and specific receptors for sensing molecules present in permissive environments (such as the presence of small molecule nutrients), as well as cortex-digesting enzymes that facilitate spore activation [50-59]. The importance of germination receptors and cortex-lytic enzymes to virulence has been demonstrated in several species. The intricate molecular signalling that enables spores to sense and respond to environments has thus dispelled the notion of bacterial spores as inert entities. An elegant example is provided by *B. anthracis*, which has evolved to express virulence factors (i.e., toxins) soon after germination. Since germination occurs in host macrophages, toxin production is required for survival inside the macrophage [38,39,41,42]. It may seem counterintuitive that spores would evolve to germinate inside phagocytes, but intracellular germination is advantageous since the host immune system can be evaded, allowing free bacterial reproduction. Once the macrophage is killed, the resulting bacterial escape can result in drastic clinical consequences manifesting in overwhelming toxemia and bacteremias [38,39,41,42,60]. Another example of germination playing a role in infection comes from *C. difficile* spores, which germinate specifically in response to bile salts and glycine present in a gut environment [50,52]. This disruption also provides a niche in which ingested *C. difficile* spores can reproduce and cause disease. In both these examples (*B. anthracis* and *C. difficile*), the spore fully germinates only in an environment that is conducive for the survival of the vegetative cells (either intracellular for *B. anthracis* or in the anaerobic environment of the gut for *C. difficile*). Furthermore, eliminar, the spore responds to germinants that are uniquely present in either the macrophage or the gut to allow germination only in the right and permissive environment [50].

While many of these spore-associated virulence factors were identified in *Bacillus* spp., it is likely that clostridial spores also possess spore-specific factors to facilitate infection. Many spore-forming species such as *C. botulinum* are professional pathogens that can cause a specific type of disease (botulism), while others such as *B. anthracis* or *C. difficile* can cause a variety of disease manifestations [26-28]. In some cases, more than one species can cause the same or very similar disease (i.e., gas gangrene caused by *C. perfringens* and *Clostridium sordellii*). In others such as *Clostridium septicum*, underlying conditions such as immunodeficiency or cancer are a prerequisite to infection [20-28]. Despite the differences in disease presentation and outcome, infection primarily occurs when spores enter the body, colonize the host, germinate and produce the toxins (along with other virulence factors). In other cases, heat-stable toxins produced in contaminated food (most notably associated with *B. cereus* and *C. botulinum*) can cause food poisoning without a concurrent infection. Spore germination involves

signalling from small-molecule germinants that diffuse across the outer spore layers and bind to receptors in the cytoplasmic (inner) membrane. The dormant spore takes up water, releases calcium ions, resumes metabolic activity and degrades the cortex to allow the emergence of the vegetative cell which in turn is capable to resume active growth and division (Figure 2B) [50,58,59].

Examples of bacterial spores as agents of mass destruction

In this section we will focus on the characteristics of four previously mentioned spore formers and the diseases they produce. One of them, *B. anthracis*, is well recognized as a Class A microorganism by the CDC [1,2,60]. Other two spore-forming, *C. perfringens* and *C. botulinum*, are considered by the CDC as Class B and C agents, respectively, because of the potential use, in food and water contamination, of the deadly epsilon and botulism toxins produced by them, respectively [24,25]. The fourth spore-forming microorganism we consider here is *C. difficile*, which although not listed by the CDC as a potential agent to be used in a bioterrorist event is considered a recently emerged superbug of public concern (see before). According with this interpretation, the NIAID clasifies *C. difficile* as a Class C priority emergen pathogen [61].

B. anthracis

This spore-forming bacterium is the cause of the disease called Anthrax [60,61]. *B. anthracis* is found naturally in soil samples spread over the entire planet, mainly in a sporulated form. Their spores are highly resistant to environmental, nutritional and chemical stresses (Figure 1). The bacterium contains a capsule that plays a key role in providing resistance of *B. anthracis* to phagocytosis [41,42]. Anthrax toxins consist of three proteins called protective antigen (PA), edema factor (EF) and lethal factor (LF) [60,61]. Natural Anthrax outbreaks occur on contact of the person with infected animals (cattle, sheep, horses and goats) or products derived therefrom (housings, hair and leather). Intestinal anthrax is due to the ingestion of contaminated undercooked or raw meat and is uncommon in developed world. The symptoms of intestinal anthrax appear 2-5 days after ingestion of contaminated undercooked meat [60]. Bacteria are transported from the bowels to mesenteric and regional lymph nodes. Oral and oesophageal ulcers can occur.

Regional lymphadenopathy and edema may rarely lead to airway compromise [41,60]. Lower intestinal disease includes lesions in the cecum and terminal ileum, haemorrhagic adenitis and occasionally massive ascites [60-62]. Nausea, vomiting, abdominal pain, hematemesis, hematochezia and sepsis are all presenting features. Early diagnosis is difficult and the mortality is high (Table 2). Another disease produced by this bacteria is cutaneous anthrax, which ocurrs when B. anthracis spores entry into exposed skin through cuts and arasions. Spores germinate in the tissue and vegetative cells quickly multiply and produce anthrax toxins resulting in local edema that would ends in a painless black eschar. The eschar normally heals in 1-3 weeks. An estimated 2,000 cases of cutaneous anthrax occur worldwide annually and result from entry of spores through skin abrasions. Of all cases of anthrax reported annually, 95% are cutaneous Anthrax and 4% are intestinal Anthrax. 20% of cases of cutaneous Anthrax and 50% of cases of intestinal Anthrax, result in death of infected patients [12,62].

A less common naturally Anthrax is called inhalator or breathing Anthrax that occurs when a person inhales or breathes Anthrax spores present in the air particles (Table 2). This type of Anthrax occurs naturally in only 1% of all cases but leads to death of the patient in 90% of cases [60]. This is precisely the kind of Anthrax that a bioterrorist would choose to attack the population of a given city or region [1,2,26,62]. The United States Working Group on Civilian Biodefense [63] and CDC identify Anthrax as one of a limited number of biological agents capable of causing death and disease in sufficient numbers to cripple a developed region or urban setting [1,62-65]. Research into the use of *Bacillus anthracis* as a bioweapon is at least 100 years old and several nations are believed to have weaponised Anthrax [2,66-68]. The accidental release of aerosolized Anthrax spores from a military microbiology facility in 1979 at Sverdlovsk, of the former Soviet Union, caused at least 68 deaths and demonstrated the lethal potential of aerosolized *B. anthracis* [69]. A previous event happened during 1941 and 1942 when England (more precisely W. Churchill, fearing Nazi Germany use weapons of mass destruction against the English people decided to develop their own biological weapons to be used in German territory as retaliation. The type of biological weapon developed by the British was aerolized Anthrax (i.e. highly concentrated spores of *B. anthracis*, packed in fine powders of no more than 5 microns in diameter). For this purpose a series of secret experiments were conducted and to determine the degree of success it was decided to test the secret weapon on the Scottish island of Gruinard which had previously been populated with lots of livestock, especially sheep. After releasing aerosols Anthrax on the island, it was noticed that the experiment was very successful and the island was highly colonized by *B. anthracis* and therefore was unsafe for animals and humans [70]. This situation of high pollution of the island continued until the early 1980s when members of an environmental group travelled to the island clandestinely and "stole" land (still highly contaminated with *B. anthracis* spores). The environmental group sent letters containing the contaminated soil to several members of the British cabinet requiring decontamination Gruinard Island under threat of spreading greater amounts of land with Anthrax among members of the British government. This "threat-blackmail" produced the desired effect and the Government of His Majesty decontaminated the island flooding it with large amounts of formamide to inactivate and kill spores [71]. Today Gruinard Island can be accessed by anyone without risk of Anthrax and represents perhaps the first example of "bioterrorist attack" but with an "environmentalist" finale. Simulation studies conducted by the World Health Organization and the United States Congressional Office of Technology Assessment together with CDC staff and the Pentagon (before the attacks of 2001) led to the conclusion that beginning with the dispersal of 1 Kg of aerosolized *B. anthracis* spores, a form of lung Anthrax would after three months to cover all the US having produced more than 100,000 dead and 3 million patients, as well as producing the collapse of the entire health system of the country as there is no in the US antibiotics enough to supply the entire population at risk of infection [1-3,62-64].

Twenty-two cases of bioterrorism-related Anthrax were identified in the United States during 2001 when tin-covered *B. anthracis* spores were delivered by mail letters of the US postal service [1-3,64-66]. There were 5 fatalities involving 11 cases of inhalational anthrax and 11 cases of cutaneous anthrax [1-3,64-66]. The mortality from untreated inhalational Anthrax approaches 100% and the costs associated with a real or perceived *B.anthracis* bioterrorist attack have been estimated at over $26 billion per 100,000 persons exposed [1-7,11-12]. For three types of Anthrax the selected treatment is antibiotic therapy, important thing is to discover the disease in its early stages. This is difficult to achieve in cases of inhalator Anthrax, because aerosolized spores, which size is between 2 and 5 μm in size, reach the alveolar ducts and alveoli [42,60]. Pulmonary macrophages ingest and transport the spores to mediastinal and hilar lymph nodes where germination and toxin production ensues. The toxins are released into the systemic circulation, resulting

Microorganism	*Bacillus anthracis*	*Clostridium botulinum*	*Clostridium difficile*	*Clostridium perfringens*	*Coxiella burnetii*
Infective form during aerolization	Spore	Spore	Spore	Spore	Small cell variant (SCV) and "pseudo-spores"
Infectious aerolized dose	5.0-10.0 spores (ID10%)	Not known	Not known	Not known	1.0-3.0 SCV (ID50%)
Activation of the infective form	Spore germinant / co-germinant: L-amino acids, proteins, inosine (present in nucleic acids, foods and creams)	Spore germinant / co-germinant: L-amino acids, proteins, L-lactate (present in foods, creams), adenosine (present in nucleic acids) and CO_2	Spore germinant / co-germinant: CO_2, biliar salts, L-amino acids, proteins	Spore germinant / co-germinant: L-amino acids, proteins, sugars (glucose and fructose), potassium, phosphate, CO_2, inosine	Phagocytosis of SCV form by macrophages and conversion to LCV
Caused disease	Pneumonic and Intestinal Anthrax	Intestinal Botulism (similar to Infant Botulism)	Bloody Diarrhoea	Bloody Diarrhoea and Gas Gangrene	Q fever
Incubation period	1 to 7 days	1 to 7 days	1 to 7 days	1 to 7 days	1 to 3 weeks
Symptoms after bioattack and agent activation	Inhalation of spores: Fever and chills, chest discomfort, shortness of breath, confusion or dizziness, coughing up blood, nausea, vomiting, or stomach pains, headache, sweats (often drenching), extreme tiredness, body aches Ingestion of spores: Fever and chills, swelling of neck or neck glands, sore throat, painful swallowing, hoarseness, nausea and vomiting, especially bloody vomiting, diarrhoea or bloody diarrhoea, headache, flushing (red face) and red eyes, stomach pain, fainting, swelling of abdomen (stomach)	Inhalation of spores: not known Ingestion of spores: Difficulty swallowing or speaking, dry mouth, facial weakness on both sides of the face, blurred or double vision, drooping eyelids, trouble breathing, nausea, vomiting and abdominal cramps, paralysis and life compromise if untreated	Inhalation of spores: not known Ingestion of spores: Watery diarrhoea, up to 15 times each day, severe abdominal pain, loss of appetite and loss weight, fever, patches of raw tissue that can bleed or produce pus (pseudomembranous colitis), kidney failure and death if untreated	Inhalation of spores: not known Ingestion of spores: a- Mild diarrhoea to a life-threatening sequence of severe abdominal pain, vomiting, bloody stool, ulceration of the small intestine with leakage (perforation) into the peritoneal cavity and possible death b- Gas gangrene (intestinal mionecrosis): breakdown of muscles, severe pain, oedema, tenderness and pallor, discoloration and hemorrhagic bullae and production of gas at the site of wound. Life compromise	Inhalation /ingestion of SCV: High fever, severe headache, general malaise, myalgia, chills and/or sweats, cough, nausea, vomiting, diarrhea, abdominal pain, chest pain. It can developed in pneumonia, granulomatous hepatitis, myocarditis and central nervous system compromise
Dissemination	- Aerosol droplets - Contact with infected animals and infected people - Contact with contaminated objects or surfaces - Diarrhoea if the attack is combined with C. difficile and/or C. perfringens	- Aerosol droplets - Contact with Contaminate objects or surfaces - Diarrhoea if the attack is combined with C. difficile and/or C. perfringens	- Aerosol droplets - Transmission from person to person - Contact with contaminated surfaces - Diarrhoea	-Aerosol droplets -Transmission from person to person - Contact with contaminated surfaces - Diarrhoea	- Aerosol droplets -Transmission from person to person - Contact with contaminated surfaces - Diarrhoea if the attack is combined with C. difficile and/or C. perfringens
Contagious from person to person in case of aerosol dissemination	Yes	Not known but plausible if the attack is combined with C. perfringens and/or C. difficile	Yes	Yes	Yes

Treatment	Antibiotics, sporocides	Antitoxins, antibiotics and sporocides. Breathing machines (ventilators) in severe cases	Antibiotics, sporocides and/or surgery	Antibiotics, sporocides and/or surgery	Antibiotics, and/or surgery
Vaccine availability	Yes, but its efficacy against pulmonary anthrax is controversial	No	No	No	Yes, but its efficacy against a high inhaled dose of SCV is not known

Table 2: Summary of the main infective properties of pathogenic Bacilli, Clostridia and *Coxiella*, the bioterrorist linked diseases and treatments and preventive actions to be taken to ameliorate illness and deaths.

in edema, haemorrhage, necrosis, septic shock and death [60]. Spore germination in the mediastinum results in haemorrhagic mediastinitis, a hallmark of inhalational anthrax. During the first week, symptoms are indistinguishable from those of a common cold but after that time spores already germinated in the lung alveoli, the emerging vegetative cells migrated to the lymph nodes and then invaded the blood to spread throughout the body. At this time appear the most characteristic symptoms in the ill person, bleeding and difficulty breathing (Table 2). The bacterial infection could be originated by the inhalation of no more than ten spores has spread throughout the body being able to reach titles of 100 million bacteria per mille litre of blood, a fact that probably results all antibiotic therapy fruitless [72-74]. There are at least two types of vaccines, one with an attenuated strain and another cell-free vaccine. In both cases six doses distributed over 18 months are required [75]. These vaccines lose protective effect over time and it is uncertain its efficacy in cases of inhalatory Anthrax, especially in case of bioterrorist attacks wherein the doses of Anthrax spores inhaled would be several times higher than those found naturally in the air (Table 2). In a worse situation, *B. anthracis* strains used for an attack could be genetically modified to further increase their virulence and antibiotic resistance or combined with other bioterrorist agents, see below [31,32,35].

C. botulinum

Although bioterrorism-linked botulism outbreaks has been almost exclusively associated with food -and water- poisoning with the potent botulism toxin [24,76,77], we envision that an intentional release of aerosols containing spores of this pathogen might reach several of the objectives of a biological attack (illness and panic) [2-4,68] (Table 1). In fact, for surveillance purposes, the CDC categorizes human botulism cases into four types: foodborne, wound, infant and other. While the first two types of human botulism are associated with the ingestion of the botulism toxin, the other two (infant and other botulisms) include botulism in which the transmission is unknown. In this sense, the "other" category of botulism includes the intestinal colonization by the bacterium with the *in situ* production of botulism toxin. Taking into consideration that in an intentional release (i.e. a bioterrorist attack), exposure may occur by routes in which the bio agent is not transmitted in nature, we think that *C. botulinum* gut colonization might occur in people that breaths aerosols containing spores of this pathogen.

Botulism is a rare (fewer than 200 cases of all forms of botulism reported annually in the United States) but serious illness caused by the different toxin (neurotoxin) produced by the vegetative form of *C. botulinum* after spore germination. All forms of botulism result from absorption of botulinum toxin into the circulation from either a mucosal surface (gut, lung) or a wound [78-80]. Botulinum toxin does not penetrate intact skin. Wound and intestinal botulism result from production of botulinum toxin by *C. botulinum* on tissue or in the intestinal lumen. A man-made form that results from aerosolized botulinum toxin or botulinum spores is inhalational botulism. Inhalational botulism has occurred accidentally in humans. All forms of human botulism display virtually identical neurologic signs that may be preceded by abdominal cramps, nausea, vomiting, or diarrhoea [78-80] (Table 2). The extent of paralysis may vary considerably among patients. Some patients may be mildly affected, while others maybe so paralyzed that they appear comatose and require months of ventilatory support. The rapidity of onset and the severity of paralysis depend on the amount of toxin produced and absorbed into the circulation. The toxin blocks excitatory synaptic transmission by inhibiting acetylcholine release causing flaccid paralysis at the neuromuscular junction [78-80]. Recovery in adults, may take weeks or months. Patients with botulism typically present with difficulty seeing, speaking, and/or swallowing. In untreated persons, death results from airway obstruction (pharyngeal and upper airway muscle paralysis).

Although commonly botulism is considered an intoxication and patients remain afebrile, it is also possible an infection produced by C. botulinum vegetative cells replication. Examples of *C. botulinum* infections are wound botulism (produced by the neurotoxin produced from a wound infected by *C. botulinum*) and infant botulism caused by consuming honey or powdered milk contaminated with spores of *C. botulinum*, which germinate and grow in the intestine with the releasing of the neurotoxin. In a case of a bioterrorist attack using aerosolized *C. botulinum* spores it might be predicted that *C. botulinum*, after spore germination, could produce an infection with release of the neurotoxin at mucosal sites (i.e. gastrointestinal tract) of injured persons (Table 2). Botulism can be treated with an antitoxin which blocks the action of the circulating toxin and in cases of *C. botulism* infections, as it might happen in case of a bioterrorist attack, antibiotics, antitoxins and sporocides will be required (Table 2) [24,81-85]. Although there are no reports on the use of *C. botulinum* spores in bioterrorism events, the spore dormancy makes spores harmless to anyone (a bioterrorist) handling them. Furthermore, the ease of handling spores in comparison with manipulation of toxins makes spores as the ideal weapons to disseminate *C. botulinum* (Table 2).

Although in the last decades, the proportion of people with botulism who die has fallen from about 50% to less than 5% (www.cdc.gov/botulism), a hypothetical bioterrorist attack with aerosolized *C. botulinum* spores, we predict medical and nursing care, the stocks of antitoxin, antibiotics and the breathing machines would rapidly turn insufficient and collapse. Besides an uncertain number of deaths in case of a bioterrorist attack with *C. botulinum* spores, the botulism survivors might require long time of supporting therapy because of the possibility of secondary infections acquired during the breathing therapy using machines and/or problems related to paralysis (Table 2).

C. difficile

Infections caused by this spore-forming bacterium belong to the group of infections called healthcare-associated infections (HAIs). *C. difficile* causes a spectrum of diseases ranging from diarrhoea to pseudomembranous colitis that would end in death [27,28]. *C. difficile* caused almost half million infections in the US in 2011, and almost 30,000 died within the first month of the infection [86,87]. In addition, *C. difficile* infections are one of the most costly nosocomial infections, responsible for estimated 3 billion dollars in increased healthcare cost annually [27,28,86,87]. *C. difficile* spores spread rapidly because of the aerosols produced with the diarrhoea in addition to the resistance of the bacteria to many drugs used to treat microbial infections and many of the disinfectants currently used in healthcare environments are inactive against *C. difficile* spores, mainly when spores are deposited on dirty surfaces [48]. In the present century, stronger strains of *C. difficile* emerged [88-91]. These strains are resistant to fluoroquinolone antibiotics, which are commonly used to treat clostridia and other infections. The ability to form spores and its, acquired resistance to common antibiotics plus toxin overproduction turn *C. difficile* in a superbug of public concern which has spread throughout North America and Europe producing a 400% increase in deaths since 2000 to 2007 [87-91]. Because of the rapid spore-mediated spreading of *C. difficile* infections, the antibiotic resistance profile of virulent *C. difficile* strains and the robustness and persistence of *C. difficile* spores in the environment, we indicate that if *C. difficile* is manipulated (i.e. aerosolized) by the wrong hands, it might represent, (under spore form to protect this anaerobic pathogen from air exposition), an ideal and novel agent of bioterrorist utilization we must take into consideration (Table 2).

C. perfringens

This spore-forming bacterium can be found in almost any environment. It is able to produce at least thirteen different toxins [92-96] and a similar number of virulent-associated factors [97-100]. It is considered the most widely distributed pathogen in nature [97]. Two diseases are commonly caused by *C. perfringens*: food poisoning and gas gangrene [98-100]. *C. perfringens* is one of the most common causes of foodborne illness in the US because of the production of a potent and sporulation-related enterotoxin named CPE (C. Perfringens Enterotoxin) [96-98]. It is estimated that it causes nearly a million cases of foodborne illness each year mainly diarrhoea and abdominal cramps [94-98]. Beef, poultry, gravies and other precooked foods are common sources for *C. perfringens* infections because they are prepared in large quantities and kept warm before consumed. *C. perfringens* spores germinate under these conditions and when they, or the newly-formed vegetative cells, start a new round of sporulation, the CPE is produced and release. People consuming this CPE-contaminated food get ill in a few hours [96-98]. A more life-threatening infection due to *C. perfringens* is gas gangrene, a highly lethal necrotizing soft tissue infection of skeletal muscle caused by toxins (mainly PLC-phospholipase and PFO-perfringolisin) and gas produced by this bacterium [95-97,100]. Clostridial gas gangrene produces death of body tissues due to a lack of blood flow produced by the bacterial toxins and the concurrent consume of the destroyed tissue as a food-source for the same bacterium. Gas gangrene most commonly affects the extremities, but it can also occur in muscles and internal organs. The chances of developing gas gangrene are higher if underlying conditions that can damage blood vessels affect blood flow, such as diabetes or atherosclerosis [101]. The absence of early radical surgery, antibiotic therapy and (if available) hyperbaric treatment, leads to the spread of toxins in the body causing shock, coma and death [101]. The association of this anaerobic bacterium with a bioterrorist threat has been associated with the production of the epsilon toxin considered to be the third most powerful bacterial toxin [25,102]. However, this pathogen represents a substrate for the production of biological weapons. If aerosolized spores of *C. perfringens* were dispersed, they could potentially induce outbreaks of food poisoning and lead to increase morbidity of gas gangrene [103]. We envision the intentional release of aerosols containing *C. perfringens* spores would allow spores inhaled by people to end up in the intestinal tract.

Based on the profile of produced toxins, *C. perfringens* is classified in different types or toxi-types [95,96]. Type A *C. perfringens* strains produce enterotoxin and alpha toxin, responsible for diarrhoea and gas gangrene, respectively, in humans. Type B and D strains usually infect animals (calves, piglets, sheep, lambs, goats, etc) but rarely humans but its use as an aerosolized weapon can change the situation. Type B and D isolates, in addition to alpha toxin, also produce epsilon toxin. Ingestion of epsilon toxin or viable spores would end up in pulmonary edema and neurologic dysfunction [96,102]. Therefore, *C. perfringens* may be weaponized either as purified epsilon toxin or as viable spores (Table 2). Although a high number of spores, 10,000-100,000 spores/g of contaminated food, are required to produce foodborne illness [94] it is not known the infectious dose for the development of a gas gangrene, diarrhoea or epsilon toxin intoxication in case of utilization of aerosolized *C. perfringens* spores (Table 2). However, it might be predicted a generalized panic that would happen if aerosolized spores responsible for gas gangrene production were disseminated among people among people, similar to the prevailing chaos during the pandemic plague that ravaged Europe in the XIV-XVI centuries [4].

A special case: *Coxiella burnetii* and Q fever

Q fever is a worldwide disease caused by the Gram-negative bacteria *Coxiella burnetii* [29,104]. Farm animals (cattle, sheep and goats) are the primary reservoirs of *C. burnetti* that is excreted with the fluids (milk, urine and faeces) of infected animals [104,105]. The bacterium is also able to reach the amniotic fluids and colonize the placenta [29,106,107]. Why is this pathogen included in this revision? *C. burnetii* produces very resistant cellular forms (named small cell variant, SCV) [30] that, without being as robust as the spores, are much more resistant and virulent (infectious dose of one SCV per person) than the vegetative forms of other dangerous bacteria such as the Class A bacterium *Yersinia pestis*, the causative agent of the Plague [108-110]. The SCV are extremely hard and resistant to heat, high pressure, drying, osmotic treatment, UV light and many biocides. These properties allow *C. burnetii* to survive for very long periods in the environment where infection of humans happens by inhalation of SCV [29,108-110].

The fact that this bacterium is an obligate intracellular pathogen prevented for a long time the consideration of *C. burnetii* as a biological weapon of first choice. However, the development of genetic tools to improve its virulence and the formulation of a host cell free culture medium [111,112] allowing the free reproduction of *C. burnetii* with formation of SCVs have placed this bacterium as very attractive for its use in the wrong hands as a biological weapon [29,108]. In humans, the disease may appear in two forms, acute or chronic. Acute Q fever may be asymptomatic or appear as atypical pneumonia or influenza-like illness [106-110]. When the immune system fails to control the acute phase, chronic Q fever emerges as hepatitis, endocarditis, osteomyelitis or aortic aneurisms (Table 2) [106,110]. *C. burnetii* is highly infectious by the aerosol route and can survive for long periods in the environment [29,105]. The cell cycle of *C. burnetii* includes the formation of the very infectious SCV of 0.2 to 0.5 μm in length and more pleomorphic large

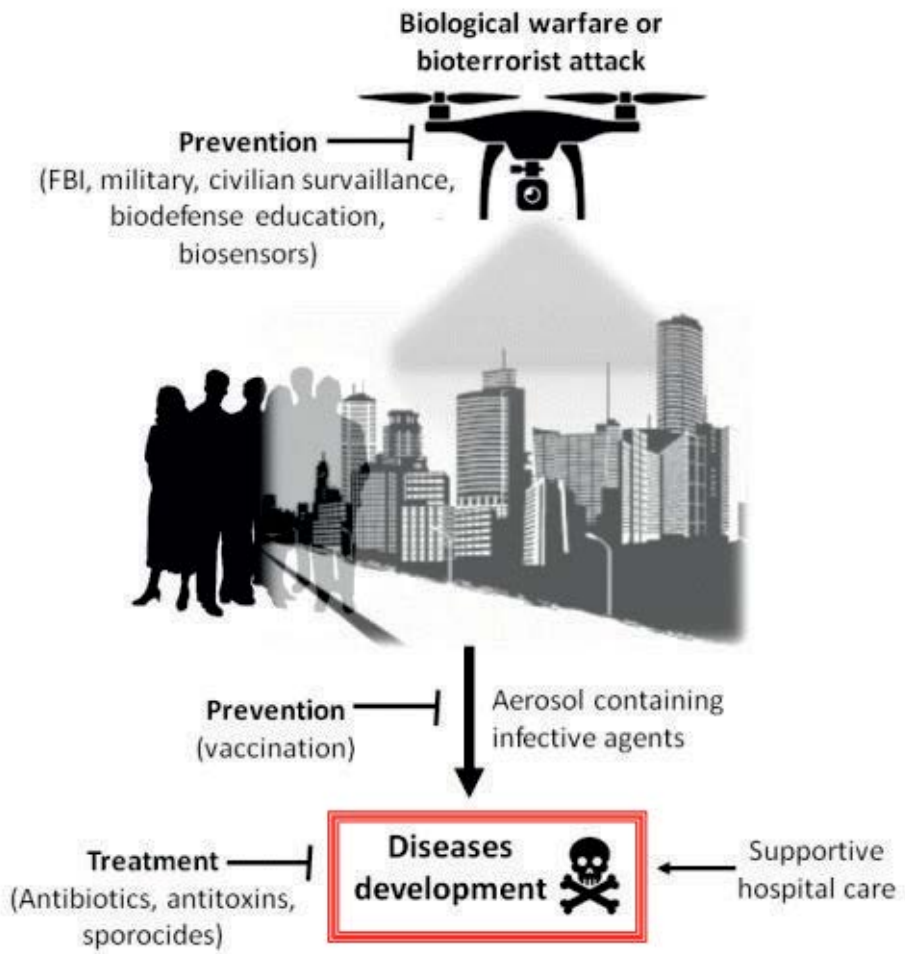

Figure 3: A hypothetical scenario showing measures that should be taken to prevent a bioterrorist attack using aerosolized spores or SCV of *BacillusClostridium* or *Coxiella burnetii*, respectively, or in the worst case mitigate its effects. See text for details.

cell variants (LCVs) of more than 1 μm in length [30]. Different reports suggested that *C. burnetii* is also able to form spores from LCVs but this claim is controversial and is not currently considered because the physical-chemical properties of the SCVs (decreased metabolism, condense chromatin and thick wall) are sufficient to account for the extraordinary stability of the agent and its environmental transmission [29-30,108,110]. It is considered that a biological attack with aerolized SCVs of *C. burnetii* will affect a large number of persons because this pathogen has been shown to travel over large distances on the winds during natural outbreaks [107,113,114]. In addition, as indicated, its infectious dose for man is extremely low [29,108-110] (Table 2).

Conclusions

Each of the described pathogens (*B. anthracis, C. botulinum, C. difficile, C. perfringens* and *C. burnetii*) is relatively easy to grow in specific culture media to allow the production of high titres of the resistant forms: spores and SCVs for Bacilli-Clostridia and *C. burnetii*, respectively. High titles of each of these pathogens are feasible to obtain after incubation for 48 h to 96 h at a temperature not lower than 25°C and not higher than 37 °C. The clostridia (*C. botulinum*, *C. perfringens* and *C. difficile*), because of their strict anaerobic growth characteristics, could become a little more difficult to grow than *B. anthracis* and *Coxiella* but a microbiology laboratory with trained staff should not have major problems in achieving growth and sporulation of *Clostridium* with high yields and rates of sporulation. In (Figure 3), we depict what would happens if a bioterrorist group would be able to grow and aerosolize the resistant forms (spores and SCVs) of the discussed pathogens at an average title of 4 x108 CFU/mL. The uniformity of the distribution of the aerosolized particles in the air will depend on various factors such as the intensity and direction of the prevailing wind at the time of the attack but we must also remember while the primary intention of a bioterrorist event is to generate the highest proportion of sick people and possible deaths, is also true that an objective of all bioterrorist event is to produce the biggest panic and fear as possible. As an example, is enough to remember the panic generated not only in the US but worldwide with the use of *B. anthracis* spores to contaminate mail letters of the US postal service during October-November 2001 that "only" produced 22 cases of Anthrax and 5 fatalities.

How to react and how to prevent this sort of aggression against humankind? Two actions are essential: efficiency in the prevention and effectiveness in the response [9-10,115-118]. The several days incubation period of the disease produced by the microorganism used in the bioterrorist attack (Table 2) makes accurate diagnosis [115-117] and rapid treatment essentials to save as many lives as

possible [8,12,118,119]. Prevention activities should involve areas of military and police intelligence, federal agencies like the FBI [7,9,11]. It is also very important to educate citizens on how to be prevented and how to act early to detect strange behaviours or activities out the ordinary [13,14]. Vaccination campaigns are very useful, especially to protect members of the military, police, civil defence and health care institutions that must act quickly and actively to heal the sick, prevent further deaths and mitigate the chaos and panic [11-12,75]. Adequate stocks and distribution capabilities of antibiotics and medicines are crucial to mitigate the post attack consequences. All these measures of prevention and response must be highly coordinated and must be periodically tested in simulated situations by professionals and by civilians [13,14]. These simulations would allow to know how rapidly a federal, state or local community recognize, respond and recover from a bioterrorist attack (Figure 3). Also important is the responsiveness of general citizens and therefore, education of citizens on how to react and what to do in case of a bioterrorist attack is very important. These programs focused on the general public would not increase their fear but inform and educate on how to manage the problem. Unfortunately, only US government and few other countries face the bioterrorist scenario as a real threat. Finally, scientists have a crucial role [31,32,66] in the development of improved technology [34,35,68] for the early detection of a bio threat (e.g. real-time biosensors) [116-117], effective vaccines [75,120,121] against aerosols containing high amounts of pathogens (e.g. mucosal vaccines), new medicines [119,122-128] and biocides and sporocides [85,129] working on biotic and dirty surfaces.

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