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# *Bacillus anthracis*, virulence factors, PCR, and interpretation of results

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Anthrax is a non-contagious infectious disease that affects a wide range of animal species, including humans, but especially sensitive are domestic and wild ruminants. The bacterial agent is *Bacillus anthracis*, whose main feature is to form spores that can survive in the environment for several decades. Anthrax, in susceptible animals, has a generally fatal outcome characterized by sudden death and leakage of blood from the 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 form (fatal). Recently a fourth fatal form (injectional anthrax) has been reported in drug users as a result of injections of heroin contaminated with anthrax spores.[1] Thanks to its strong ability to maintain the vitality and virulence for many decades and to the very low costs of production, *B. anthracis* is considered one of the pathogens of interest such as bacteriological weapon in a hypothetical bioterrorist attack.

*B. anthracis* spends most of its existence the ground as a spore, until the ideal conditions are created, allowing it to initiate a reproductive cycle that occurs in a different habitat represented mainly by domestic and wild ruminants. Nature provides few opportunities to the bacterium for its replication cycle, and the development of an extraordinary virulence is the effective strategy to significantly increase the probability of success against the host immune mechanisms. The rapid and intense multiplication of vegetative cells within the host brings it to a speedy death. Although many of the new generations of bacteria are neutralized by putrefactive processes, a sufficient part of them survive and spread into the surrounding soil as spores. The bacterium acts within its host with an exceptional virulence because its objective is to kill the host and produce a large amount of spores able to guarantee the standard of environmental density essential for the continuation of the species. Reported are cases of animals that survive the disease, but these could represent a failure of the bacteria because this try doesn't produce spores. In summary, the few animal anthrax cases that occur each year in endemic areas of the world are nothing more than the result of a natural ecological balance that through these extraordinary events seeks to promote the maintenance of a bacterial species that otherwise would have become extinct long time ago (personal consideration).

Virulent forms of *B. anthracis* harbor two large pathogenicity-related plasmids: pXO1 (181.6 kb), which encodes the anthrax toxin genes *pagA*, *lef*, and *cya*[2,3] and pXO2 (93.5 kb), which carries the genes responsible for capsule synthesis and degradation, *capA*, *-B*, *-C*, and *-D*.[4-8] *B. anthracis* expresses its pathogenic activity mainly through the capsule (anti-phagocytic activity) and the production of a toxic complex consisting of three proteins known as protective antigen (PA), lethal factor (LF), and edema factor (EF). The two large plasmids of *B. anthracis* are essential for full pathogenicity; elimination of either dramatically attenuates the virulence of *B. anthracis*

Following the bioterrorist attacks took place in autumn 2001 in the United States, western countries have invested heavily in research on highly pathogen agents, and in particular on *Bacillus anthracis*. However, in the constant race in search of increasingly sophisticated molecular diagnostic methods, it must be remembered that there is still no selective medium for *Bacillus anthracis*. Therefore, it is necessary that the scientific world pay more attention in the search for microbiological diagnostic systems easily able to isolate the pathogen agent from biological and environmental samples.

The problem regarding the biomolecular approach for the identification of *Bacillus anthracis* is its strong similarity with other strains in the genus *Bacillus* and in particular with certain strains of *B. thuringiensis* and *B. cereus* that are genetically very close to *B. anthracis* cluster.[9,10] The main difference between these two groups is the presence of the two plasmids of virulence. Almost all of the biomolecular identification test for *B. anthracis* is based on primers and probes specific for chromosome and the two plasmids. However, there are strains of *B. anthracis* with faults in their genetic pattern. The Sterne strain, which is devoid of plasmid pXO2, is capable of producing toxins but does not survive within the host for a long time as the lack of its capsule makes it easily attacked by phagocytic processes. Conversely the strain Pasteur, while having a capsule, should be able to avoid phagocytic process, but, not having the plasmid pXO1, is unable to produce toxic factors and thus is practically harmless. In the natural environment the loss of the plasmid pXO1 is rare while the loss of pXO2 is

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common.[11] Moreover, the simultaneous presence of both plasmids is not sufficient to define the virulence of a strain, such as the attenuated vaccine strain "Carbosap", which, despite presenting both plasmids, is apathogenic for the rabbit and retains residual pathogenicity for mice and guinea pigs.[12] Anomalies are also present in some strains of *B. cereus*. The most famous is the case related to two strains of *B. cereus* isolated from dead monkeys in the tropical forests of Ivory Coast and Cameroon with an anthrax-like form, which are equipped with two plasmids very similar to pXO1 and pXO2 of *B. anthracis*.[13,14] Due to the high homology in the chromosome DNA, cross-reactivity between *B. anthracis* and other strains of the genus *Bacillus* are frequent in the chromosome target for PCR. For this reason it is important to have specific tests for the chromosome target of *Bacillus anthracis*. The article in this issue of *Virulence* by Ågren et al.[15] offers a great contribution to the solution of this problem. For the detection of chromosomal DNA, the authors compared published primer and probe sequences for specificity against 134 available *Bacillus* spp. genomes. Of the 35 investigated PCR assays, only 4 were 100% specific for the anthrax chromosome. The use of a specific chromosome target offers the guarantee of a valid identification of the bacterium, especially in environmental investigations, where there is the possibility to isolate strains of *B. anthracis* that have lost both plasmids.

However, the question of the interpretation of the epidemiological significance in case of isolation of strains of *B. anthracis* devoid of one or both of the two plasmids remains open. While the isolation of strains of "apathogenic" *B. anthracis* in rural areas where animal outbreaks have occurred in the past would suggest of a natural biological phenomenon, the meaning is different when the recovery of apathogenic strains occurs on samples or places where *B. anthracis* should not be without the human hand.

Having a PCR screening test highly specific for the chromosome target can be very useful in the course of bioterroristic investigations because nothing should be neglected. The discovery of apathogenic ($pXO1^-/pXO2^-$) strain of *B. anthracis* in unusual places could be linked to a demonstrative act and can demonstrate the ability of the responsible group for knowing how to manipulate strains of anthrax, as happened in 1993 when the Aum Shinrikyo cult performed a bacteriological attack using a Sterne strain.[16]

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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