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Review

Detection technologies for *Bacillus anthracis*: Prospects and challenges ☆

Shilpakala Sainath Rao, Ketha V.K. Mohan, Chintamani D. Atreya *

Section of Cell Biology, Laboratory of Cellular Hematology, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, United States

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ABSTRACT

Bacillus anthracis is a Gram-positive, spore-forming bacterium representing the etiological agent of acute infectious disease anthrax, a lethal but rare disease of animals and humans in nature. With recent use of anthrax as a bioweapon, a number of techniques have been recently developed and evaluated to facilitate its rapid detection of *B. anthracis* in the environment as well as in point-of-care settings for humans suspected of exposure to the pathogen. Complex laboratory methods for *B. anthracis* identification are required since *B. anthracis* has similarities with other *Bacillus* species and its existence in both spore and vegetative forms. This review discusses current challenges and various improvements associated with anthrax agent detection.

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1. Introduction

Bacillus anthracis rarely infects humans. The ecological cycle of this Gram-positive bacterium involves proliferation of vegetative bacilli in the host and subsequent generation of dormant spores that remain viably persistent in the soil for decades. Spores ingested by grazing animals typically constitute the infectious form of this pathogen.

The virulence factors of *B. anthracis* consist of a toxin and an antiphagocytic capsular polypeptide (Green et al., 1985; Mikesell et al., 1983). The genes for these virulence factors are located on two separate plasmids, pXO1 and pXO2 (Read et al., 2003). Loss of either plasmid results in strain attenuation (Spencer, 2003). Plasmid pXO1 harbors the structural genes for the anthrax toxin proteins: edema factor [*cya*], lethal factor [*lef*] and protective antigen [*pagA*]. These proteins are individually non-toxic, but when acting in binary combinations (binary toxins) they cause edema and cell death in the host (Barth et al., 2004; Mogridge et al., 2002). Additionally, two regulatory genes *atxA* and *pagR*, and a recently characterized operon containing three genes whose functions appear to affect germination are located on pXO1.

The second plasmid pXO2 is smaller in size compared to pXO1 and carries three genes, *capB*, *capC* and *capA* essential for capsule synthesis. *Cap* polypeptide is necessary for polyglutamate capsule

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* Corresponding author. Bldg 29A, Room 2C-11, NIH campus, 8800 Rockville Pike, Bethesda, MD 20892, United States. Tel.: +1 301 827 1824; fax: +1 301 827 1825.

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synthesis (Candela et al., 2005). The capsule is composed of a polypeptide, poly-D-glutamic acid and prevents phagocytosis of vegetative forms and opsonization of the bacilli by the virtue of its sheer negative charge. A depolymerase encoded by the *dep* gene that catalyzes the hydrolysis of the poly-D-glutamic acid into lower molecular-weight polyglutamates inhibits the host defense mechanisms (Makino, 2002).

The life cycle of *B. anthracis* can be divided schematically into two phases: 1) multiplication in the mammalian host and 2) persistence in the soil (Santelli et al., 2004). Inhaled endospores are taken up either by alveolar macrophages or pulmonary dendritic cells, germinate in the phagolysosome of these immune cells and become vegetative forms of bacteria. They are then transported to local lymphatics and mediastinal lymph nodes, where they germinate and cause hemorrhagic lymphadenitis. Vegetative bacilli further spread via the bloodstream and lymphatics causing septicemia. The large amounts of toxin produced by the bacilli, together with the host response such as release of tumor necrosis factor and interleukin-1 are responsible for the rapid decline and overt symptoms of the host. If untreated, patients die within a few days.

B. anthracis ranks high on the list of potential agents of bioterrorism. *B. anthracis* forms large Gram-positive rods. Cells of this facultative anaerobe are non-motile, encapsulated, and arranged in chains. They form spores that are resistant to harsh environments including ultraviolet light, ionizing radiation, heat and various chemicals. The vegetative form is not readily transmissible but the spores can be transmitted easily to humans. Recent bioterrorism events have emphasized the need for rapid and accurate detection and identification of *B. anthracis* as part of 'Emergency Preparedness' to protect public health. Various tests have been developed to detect and identify anthrax. Although many of these technologies supposed to be rapid, accurate and reliable, only a few have been extensively evaluated under field conditions.

2. Challenges in the detection of *B. anthracis*

An ideal detection system would be one that detects very low copy number of organisms (sensitivity), with no cross-reactivity (specificity), takes short time for the assay, is simple to perform (not requiring trained personnel) and cost-effective. Like with any microbial detection technology, there are major challenges in developing a fool-proof detection system for *B. anthracis* as well.

The major setback with *B. anthracis* detection stems from its similarity with other strains in the genera, which leads to the identification of false-positives. *B. anthracis* forms a highly monomorphic lineage within the *B. cereus* group, but strains of *B. thuringiensis* and *B. cereus* do exist that are genetically closely related to the *B. anthracis* cluster. The main difference between the two groups is the presence of two virulence plasmids in *B. anthracis*, and plasmids coding for insecticidal toxins in *B. thuringiensis*. The Pasteur and Sterne vaccine strains of *B. anthracis* lack either the toxin plasmid pXO1 or, the capsule plasmid pXO2 respectively. In another attenuated *B. anthracis* vaccine strain, the Italian 'Carbosap' a plasmid pattern similar to atypical Pasteur vaccine strains was observed and interestingly both pXO1 and pXO2 plasmids are present in this strain, which incidentally also demonstrates residual pathogenicity in mice and guinea pigs (Fasanella et al., 2001). Loss of pXO1 in environmental samples is rare, while loss of pXO2 is common (Mock and Fouet, 2001). Without the insecticidal genes, *B. thuringiensis* is indistinguishable from *B. cereus* (Helgason et al., 2000). Also, there is some argument for the possibility that the three members of the *B. cereus* group are in fact the same species with varying plasmids resulting from horizontal genetic exchange (Helgason et al., 2000).

Interestingly, a series of findings suggest that the presence of pXO1 and pXO2 no longer principally separates *B. anthracis* from other *Bacilli*. In 1994, a *B. cereus* strain G9241, that was associated with severe pneumonia in a welder from Louisiana was isolated and shown to carry an almost complete pXO1 plasmid. This strain also had a capsule, but the capsule was not composed of D-glutamyl polypeptides and was not encoded by *B. anthracis cap* genes located on the *B. anthracis* pXO2 plasmid. Similarly, in October and November 2003 two more strains of *B. cereus* causing pneumonia were isolated from workers in Texas (Hoffmaster et al., 2004, 2006). More recently, two *B. cereus* group strains, CI and CA, carrying two plasmids with close identity to *B. anthracis* pXO1 and pXO2 were recovered from great apes that had died due to anthrax-like disease in tropical forests of Ivory Coast and Cameroon. The pXO1 and pXO2 plasmids in these strains carried the anthrax toxin genes and the polyglutamate capsule biosynthesis genes respectively along with the associated regulatory genes (*atxA, acpA*, and *acpB*) (Klee et al., 2006; Kolsto et al., 2009). The *B. anthracis* lineage carries a specific mutation in the global regulator *PlcR*, which controls the transcription of secreted virulence factors in *B. cereus* and *B. thuringiensis*. It is believed that co-evolution of *B. anthracis* chromosome with its plasmids may form the basis for the uniqueness of the *B. anthracis* lineage (Kolsto et al., 2009).

Recent studies have focused on finding differences between *B. anthracis* and related species (Blackwood et al., 2004; Daffonchio et al., 1999; Patra et al., 1996; Qi et al., 2001; Radnedge et al., 2003). Since the *B. cereus* group consists of *B. anthracis, B. cereus*, *B. mycoides*, *B. thuringiensis, B. pseudomycoides* and *B. weihenstaphenensis* the 16 S rRNA and 23 S rRNA is very similar between these organisms (Ash et al., 1991; Ash and Collins, 1992). However, the differences become evident with the 16 S–23 S internal transcribed spacer sequences wherein a single nucleotide deletion between *B. anthracis and B. cereus* and a 13-nucleotide difference between *B. anthracis* and *B. mycoides* has been reported (Bourque et al., 1995a,b; Edwards et al., 2006; Harrell et al., 1995). *B. mycoides* differs from the other *B. cereus* group with regard to morphological differences (e.g., lack of motility and rhizoid colonies by *B. mycoides* on agar) though loss of the ability to form rhizoid colonies is often observed during laboratory culture. Differentiating *B. anthracis* from *Bacillus* sp. in the other *B. subtilis* group (comprising of *B. subtilis, B. megaterium* and *B. atrophaeus*) is not a problem as significant sequence variability exists within the intergenic spacer region between the 16 S and 23 S rRNA. Furthermore, structural carbohydrate profiles reveal substantial differences between the *B. cereus* group and *B. subtilis* indicating them to be taxonomically different (Wunschel et al., 1994). Differentiation within the *B. subtilis* group is also comparatively straightforward with substantial changes existing between *Bacillus* sp. in the group. Recently, *B. subtilis* has been divided into two subgroups of strains related genotypically and phenotypically to the widely studied strains 168 and W23. The 168 group was designated as *Bacillus subtilis* subsp. *subtilis* subsp. nov. and the W23 group was designated as *Bacillus subtilis* subsp. *spizizenii*, subsp. nov. and 99.5% sequence similarity was observed between the 16 S rRNA genes of the 168 and W23 groups (Nakamura et al., 1999). ISR variability comparisons between *B. subtilis* and *B. atrophaeus* suggested that *B. atrophaeus* was a separate but closely related species to *B. subtilis* (Nakamura et al., 1999; Nagpal et al., 1998). Electrospray quadrupole mass spectrometry (ESI-Q-MS) determination of the 16 S–23 S rRNA also showed differences between these two closely related species (Johnson et al., 2000).

Besides these, small acid soluble proteins (SASPs) found in *B. anthracis* spores were used as protein markers to differentiate between *Bacillus* species (Pribil et al., 2005). SASP β was used in discriminating *B. anthracis* from *B. cereus* (Callahan et al., 2008; Castanha et al., 2006a, 2007). However *B. thuringiensis* showed similar characteristics to *B. cereus*. SASP α and β analyses showed *B. mycoides* group 2 and *B. weihenstephanensis* were distinct from the *B. cereus* group. Also SASP α-β analyses showed differences between *B. mycoides* and *B. weihenstephanensis* (Callahan et al., 2009).

DNA homology studies of different *B. anthracis* strains indicate that the organism has an inter-strain similarity of greater than 90%, and