Appl Microbiol Biotechnol (2012) 93:1411–1422
DOI 10.1007/s00253-011-3845-7

MINI-REVIEW

Rapid detection methods for *Bacillus anthracis* in environmental samples: a review

Léonid M. Irenge · Jean-Luc Gala

Received: 19 September 2011 / Revised: 12 December 2011 / Accepted: 14 December 2011 / Published online: 20 January 2012

Abstract *Bacillus anthracis* is a Gram-positive, spore-forming bacterium, which causes anthrax, an often lethal disease of animals and humans. Although the disease has been well studied since the nineteenth century, it has witnessed a renewed interest during the past decade, due to its use as a bioterrorist agent in the fall of 2001 in the USA. A number of techniques aimed at rapidly detecting *B. anthracis*, in environmental samples as well as in point-of-care settings for humans suspected of exposure to the pathogen, are now available. These technologies range from culture-based methods to portable DNA amplification devices. Despite recent developments, specific identification of *B. anthracis* still remains difficult because of its phenotypic and genotypic similarities with other *Bacillus* species. Accordingly, many efforts are being made to improve the specificity of *B. anthracis* identification. This mini-review discusses the current challenges around *B. anthracis* identification, not only in reach-back laboratories but also in the field (in operational conditions).

Keywords *Bacillus anthracis* · Rapid detection · Environmental samples · Culture · Molecular · Affinity-based methods

L. M. Irenge · J.-L. Gala
Defence Laboratories Department, Belgian Armed Forces, Brussels, Belgium

L. M. Irenge · J.-L. Gala (✉)
Centre for Applied Molecular Technologies,
Université catholique de Louvain,
UCL/30.46, Clos Chapelle-aux-Champs 30,
1200 Brussels, Belgium
e-mail: jean-luc.gala@uclouvain.be

Introduction

Bioterrorism can be defined as the deliberate release of virulent biological agents with the aim of causing illness or death in people, animals, or plants. Terrorists may use biological agents because they can be extremely difficult to detect and do not cause illness for several hours to several days (CDC 2006). Anthrax attacks on American soil in the aftermath of September 11th 2001 (Jernigan et al. 2002) resulted in many casualties and gave to the world a glimpse of the damage a large-scale bioterrorism attack could inflict to the world community, with respect to mortality, morbidity, and psychological trauma, especially in our high pace traffic world. These attacks underscored the needs for rapid and accurate methods for identification of biological warfare agents in order to allow implementation of timely relevant measures aimed at mitigating the impact of their release by containing their spread in hosts (humans or in animals). While commendable effort has been made to develop rapid identification methods for bioterrorist agents, information regarding their performance, especially on environmental samples, allegedly is scarce. This paper is a brief overview of the current available methods for identification of *Bacillus anthracis* in environmental samples. This choice stems from the fact that *B. anthracis* ranks high on the list of potential agents of bioterrorism (Zilinskas 1997; Thavaselvam and Vijayaraghavan 2001). Identification of *B. anthracis* (as well as other biowarfare agents) in environmental samples is still a real challenge.

Indeed, since more than a decade, scientists have been striving to find methods that could detect as earlier as possible the presence of anthrax spores in environment before they could cause disease and ultimately death in

humans and/or animals. These methods are badly needed by decision makers and first-line responders, who are often requested to detect rapidly the presence of *B. anthracis* spores in environmental samples.

An ideal identification method for detection of *B. anthracis* spores would be one that detects very low copy number of organisms per assay (sensitivity), with no cross-reactivity with any other closely related biological agent likely to be found in environmental samples (specificity), takes short time for the assay (swiftness), is simple to perform (not requiring highly trained personnel), with a very low hazard-risk, and is cost effective. The equipment and reagents should enable rapidly deployable identification in the field. Accordingly, they should be easy to carry and to handle and able to sustain constraining climate conditions in terms of humidity and temperature.

Bacillus anthracis and its pathogenic mechanisms

Bacillus anthracis is a Gram-positive rod-shaped and spore-forming bacterium, which causes anthrax, a serious and often fatal disease of grazing animals and occasionally humans (Dixon et al. 1999; Mock and Fouet 2001; Inglesby et al. 1999). *B. anthracis* spores are stable for long periods in soil and grazing animals are their readily available host. The disease begins with the introduction of *B. anthracis* spores into the host organism, usually through minor abrasions of the skin or insect bites (cutaneous anthrax), through minor abrasions of the gastrointestinal tract (gastrointestinal anthrax) or through inhalation (inhalation anthrax). The spores are phagocytized by macrophages and routed to regional lymph nodes where they germinate into vegetative cells (Mock and Fouet 2001). These cells multiply and subsequently disseminate via bloodstream to many distant organs, such as the spleen and lungs, and cause virulence by producing the anthrax toxins as well as the capsule. The anthrax toxins are secreted as three distinct proteins: the protective antigen (pag), the lethal factor (lef), and the edema factor (cya). The genes encoding these proteins are located on the pXO1 plasmid, and their transcription is regulated by another gene also located on pXO1 and known as the anthrax toxin activator (atxA) (Dixon et al. 2000).

None of these proteins is toxic by itself. Only two binary combinations allow the formation of toxins. Pag protein combines with lef protein and/or cya protein to form the lethal toxin (LeTx) and the edema toxin (EdTx), respectively (Mock and Mignot 2003; Guichard et al. 2011). The capsule is located outside the S layer, a layer that is directly in contact with the outside face of the peptidoglycan layer (Mesnage et al. 1997; Mesnage et al. 1998). The capsule is a polymer of D-polyglutamic acid (Ezzell and Welkos 1999; Mock and Fouet 2001). The genes required for its synthesis are located in the *capBCADE* gene cluster on the pXO2 plasmid (Dixon et al. 2000; Mock and Fouet 2001; Panucci et al. 2002). The capsule plays a major role in pathogenicity, by protecting the vegetative cells of *B. anthracis* against phagocytosis, thus enabling the bacteria to evade the host defenses (Makino et al. 1993; Mock and Fouet 2001). Spores of *B. anthracis* have an additional layer, which is not present in vegetative cells. This structure, called exosporium, is the outermost structure of the spore. It is constituted of carbohydrates and several glycoproteins (Charlton et al. 1999; Garcia-Patrone and Tandecarz 1995). While the exosporium does not seem to be involved in *B. anthracis* virulence, it plays however an important role in immune protection against anthrax as it carries various antigens (Brossier et al. 2002; Cohen et al. 2000; Mock and Fouet 2001). The immunodominant antigen within the exosporium is called the *Bacillus* collagen-like protein of *anthracis* (BclA). The BclA core contains a collagen-like region (CLR). The CLR is a highly polymorphic stretch of amino acids; it consists of 70 triplet repeats of amino acids (GXX), which is similar to the structure of mammalian collagens (Sylvestre et al. 2002; Leski et al. 2009). It has recently been shown that two oligosaccharides are linked to BclA through attachment to several amino acids within the CLR (Daubenspeck et al. 2004). The BclA plays an important role in the association of the exosporium to the extracellular matrix proteins of the host (Brahmbatt et al. 2007; Kuehn et al. 2009), which is the first step of introduction of *B. anthracis* within its host.

Challenges for identification of *B. anthracis*

The overriding concern for *B. anthracis* spore identification in environmental samples is the presence of non-virulent *Bacillus cereus* closely related to *B. anthracis*. It is indeed challenging to discriminate these isolates from *B. anthracis*, owing to numerous common phenotypical and genotypical characteristics. The fact that these close neighbors are ubiquitous in the environment further complicates identification of *B. anthracis* (Ash et al. 1991; Beyer et al. 1995; Hoffmaster et al. 2004; Hu et al. 2009; Keim et al. 1997; Merrill et al. 2006; Kuske et al. 2006; Léonard et al. 1998; Marston et al. 2006). The challenge posed by the coincidental or non-coincidental presence of pathogenic microorganisms together with closely related nonpathogenic microorganisms extends beyond *B. anthracis* and encompasses other biological agents, including organisms such as *Francisella* (Barns et al. 2005; Merrill et al. 2006), *Brucella* (Elsaghir and James 2003; Velasco et al. 1998; Scholz et al. 2008), or *Yersinia* (Whittaker 2009). Although this might be considered by many as a pure academic discussion, this evidence underscores the challenges related to a robust non-ambiguous and specific identification of biological warfare or bio-threats agents in the environment.

Current available *B. anthracis* identification methods include conventional microbiological methods, biochemical methods, and affinity- and nucleic acid-based detection methods. Each method has its own advantages and drawbacks, as discussed hereafter.

Current *B. anthracis* identification methods

Conventional methods

Conventional microbiological methods have been considered for decades as the gold standard for identification of *B. anthracis*. They are based on morphological features, which are observed after growth and staining of microorganisms. *B. anthracis* is a Gram-positive, non-motile, non β-hemolytic on sheep or horse-blood agar plates, sensitive to penicillin and susceptible to lysis by gamma phage (Turnbull 1999). *B. anthracis* selectively grows on polymyxin-lysozyme EDTA-thallous acetate agar (Knisely 1966), though this method requires an incubation period of 1–2 days and is still subject to further confirmatory tests (Titball et al. 1991). Colonies of capsulated *B. anthracis* appear in mucoid form and the capsule can be seen with McFadyean's polychrome methylene blue staining (Spencer 2003) or highlighted with Indian ink (Spencer 2003; Hoffmaster et al. 2004). Inoculation of cultured bacteria in guinea pigs or mice and observation of death might be performed to assess the agent virulence (Titball et al. 1991; Turnbull 1999).

While identification of *B. anthracis* by conventional methods is straightforward for an experimented bacteriologist, many significant drawbacks preclude the method from being used on environmental samples. As observed during the Amerithrax investigation, *B. anthracis* vegetative cells cultured from environmental samples can display phenotypic features somewhat different to those expected (Rasko et al. 2011). Moreover, some borderline environmental *B. cereus* isolates can exhibit one or many of these phenotypic features, such as lack of hemolysis, penicillin sensitivity, non-motility, and phage susceptibility (Klee et al. 2006a, b). Conversely, *B. anthracis* isolates displaying some purported *B. cereus* features, such as hemolysis, have also been reported (Klichko et al. 2003). Dealing with environmental samples may therefore prove to be difficult for first-line responders who would like to identify *B. anthracis* with conventional methods. On an operational point of view, traditional methods tend also to be labor-intensive and slow, often requiring one to 2 days of additional testing to confirm the presence of *B. anthracis* from growing colonies (Inglesby et al. 1999). Laboratory personnel also require additional training and certification to accurately identify *B. anthracis* under working conditions that meet biological safety level (BSL-3) requirements. The fact is that conventional detection approaches work better when *B. anthracis* is present in high concentrations, which is by the way the case when a patient is presenting with pulmonary anthrax and sepsis. These conditions are lacking when dealing with environmental samples. Moreover, when isolates are suspected of being *B. anthracis*, they must be further characterized by biochemical or molecular techniques to confirm unambiguously the pathogen identity. Conventional microbiological identification of *B. anthracis* spores in environmental samples, like soil samples, would prove difficult if a few pathogenic *B. anthracis* isolates are overwhelmed by large amounts of other *Bacillus* spores, some of them having the capacity to display genuine "*B. anthracis* features."

The recent discovery of new isolates of *B. cereus* causing an anthrax-*like* disease in animals while lacking *B. anthracis*-specific phenotypic characteristics (Hoffmaster et al. 2004; Klee et al. 2006a, b) is radically changing the widespread old belief that anthrax disease is restricted to the monomorphic *B. anthracis* group. Most authors actually agree that these isolates are not *B. anthracis*, despite causing an anthrax-like disease. Accordingly, the growth of *B. cereus* isolates from biological or environmental samples, in a context of an anthrax-like disease, must trigger additional tests for virulence assessment. These steps include capsule visualization by McFadyean's polychrome methylene blue staining and/or Indian ink and detection of the anthrax toxins from growing vegetative cells, using immunological assays (Swanson and Fosnocht 2000; Sastry et al. 2003; Hoffmaster et al. 2004; Guglielmo-Viret et al. 2011) or other sensitive methods such as matrix-assisted laser-desorption time-of-light mass spectrometry (Boyer et al. 2011). However, specificity and sensitivity of available anthrax toxins assays on these *B. cereus* should be assessed, as there are few differences in amino acids between toxin proteins from *B. anthracis* and from *B. cereus* causing an anthrax-like disease.

Biochemical identification methods

Biochemical pattern recognition can be used for identification of biological agents. The biochemical identification methods are used, mainly as confirmatory methods on isolated colonies already suspected of being *B. anthracis* based on their morphological characteristics. Actually, many of them consist in automated identification systems, which use pattern recognition systems based on metabolic characteristics of isolates and their susceptibility to antimicrobial agents (Baillie et al. 1995).

Other biochemical identification methods include the use of gas chromatography to determine the cellular fatty acid profile (AOAC International 2004; Song et al. 2000). Evidence is lacking on whether discrimination of *B. anthracis* with its closely related neighbors will always be clear cut (Baillie et al. 1995).

Intact cell mass spectrometry has been suggested as a rapid and reliable technique for bacterial identification. This technique relies on the reproducible detection of microbial proteins patterns. An unknown sample spectrum pattern is compared to protein patterns in the reference library, and a score for each comparison is generated. This identification process requires the compilation of large databases of bacteria reference spectra (Keys et al. 2004). The technique has been successfully used to specifically identify *B. anthracis* isolates (Lasch et al. 2009)

As discussed earlier for conventional methods, these assays are difficult to use on environmental samples because they require a growth step on culture in order to obtain bacterial isolates to be further processed. Therefore, if we consider a use in an operational setting, i.e., with a mobile laboratory in the field, these methods appear to be too time consuming, mainly because of this preliminary culture requirement. Their usefulness may also be limited by quality and completeness of available databases, which requires indeed a regular update to maintain the value of the tool. In addition, well-trained, experienced technicians are required to accurately identify or to carry out complementary testing if needed. Furthermore, the need for size-limited equipments able to sustain the transportation and weather constraints make these analytical systems unsuitable for anthrax spores identification in the field.

Affinity-based identification methods

Affinity-based assays use ligands (antibodies, peptides, or aptamers) that bind specifically to biological agents. Antibody-based assays are the most currently used for rapid detection of *B. anthracis* spores. Antibodies are raised either against the exosporium of spores, the vegetative cells, or the toxin proteins. Antibodies directed against the oligosaccharide epitopes of *B. anthracis* BclA glycoprotein are available (Mehta et al. 2006; Tamborrini et al. 2006; Kuehn et al. 2009) and have been used in various assays for detecting anthrax spores, such as: flow cytometry assay using fluorescein-labeled antibodies (Stopa 2000; Dang et al. 2001) resonance energy transfer flow cytometry assay (Zahavy et al. 2003), sandwich-based assay (Hang et al. 2008), bead-based immunoassay (Tamborrini et al. 2010), magnetic particle fluorogenic immunoassay (Yu 1998), and enzyme-linked immunosorbent assay (ELISA) assay (Kuehn et al. 2009). Antibodies to the poly-D-glutamic capsule (Taitt et al. 2002) or to the extracellular antigen 1 (EA1) (Wang et al. 2009) of the S-layer have also been raised and used for identifying *B. anthracis* spores. In addition to the detection of the entire organism, there are immunoassays combining the detection of the three toxin proteins (Swanson and Fosnocht 2000; Sastry et al. 2003).

In practice, only affinity-based methods using antibodies as ligands are commercially available. These assays are mostly designed as solid-support platforms (Biagini et al. 2004, 2005; Hindson et al. 2005). These platforms are easy to handle and yield results within minutes. Whereas affinity-based detection methods may bring an interesting contribution to the medical diagnostic and the epidemiological study of anthrax, their value for testing environmental samples is considerably hampered by a low sensitivity (Lim et al. 2005) and low specificity (Tengelsen et al. 2002). In a up to now unique peer-reviewed paper assessing the performance of immunodetection of anthrax spores, King et al. (2003) showed that detection limit these assays did not to exceed 10^5–10^6 spores, a threshold significantly and markedly higher than the detection limit range of 10^2–10^5 *B. anthracis* spores claimed by manufacturers. Regarding the specificity of these assays, caution is required, as *B. anthracis* antibodies can also cross-react with other *B. cereus* strains (Tamborrini et al. 2009). In this respect, it is of note that EA1 antigen-specific antibody, which was originally used as a *B. anthracis*-specific marker, was later proven to be a residual contaminant of spore preparations rather than a genuine component of *B. anthracis* spores (Williams and Turnbough 2004). Consequently, the development of more sensitive and specific immunoassays by improving antibodies quality and assay parameters might impact positively on the quality and speed of anthrax spore identification by first-line responders.

Nucleic acid-based detection methods

Nowadays, nucleic acid-based detection assays are increasingly becoming the cornerstone of a reliable, quick, and sensitive identification of *B. anthracis* spores. This interest stems from the fact that these techniques can detect fewer microorganisms in a short period of time. These methods rely on the selection of specific *B. anthracis* nucleic acid sequences (primers and/or probes) that are carefully selected to avoid any cross-reaction with other related species during PCR amplification (end-point and/or real-time PCR). The first nucleic acid assays developed for detection of *B. anthracis* targeted virulence genes located on plasmids pXO1 and pXO2 (Ryu et al. 2003; Cheun et al. 2003). While these targets seem to be restricted to virulent *B. anthracis* and *B. cereus* causing an anthrax-like disease (Hoffmaster et al. 2004, 2006; Klee et al. 2006a, b, 2010), the few point mutations noticed between sequences from these *B. anthracis* and *B. cereus* should be considered when setting up PCR assays in order to decrease the likelihood of false negative results. Moreover, pXO1- and pXO2-like plasmids can also be present in various non virulent environmental *B. cereus*. These plasmids are so called because they display a close relationship to pXO1 and pXO plasmids, as shown by comparative analysis alignments

(Rasko et al. 2004, 2007; Pannucci et al. 2002; Van der Auwera et al. 2008). In order to improve the specificity of *B. anthracis* detection, chromosomal targets restricted to *B. anthracis* and to some closely related *B. cereus* have been used in the assay. Chromosomal targets are potentially useful specific markers for *B. anthracis*, but their selection has proven to be a very difficult task, owing to the great genetic similarity between *B. anthracis* and *Bacillus* from the *cereus* group (Ash and Collins 1992; Harrell et al. 1995; Helgason et al. 2000; Read et al. 2003; Blackwood et al. 2004; Radnedge et al. 2003; Keim et al. 2009; Olsen et al. 2007). Consequently, single nucleotide polymorphisms (SNPs) originally thought to discriminate between *B. anthracis* and other *Bacillus* of the *cereus* are sometimes found in both species. In that respect, the BA813 sequence (Patra et al. 1996), initially reported to be *B. anthracis* specific, was later found in *B. cereus* that are closely related to *B. anthracis* (Patra et al. 1998; Ramisse et al. 1999). Another chromosomal marker (BA-5449) initially claimed to be *B. anthracis* specific (Volokhov et al. 2004) is also present in some *B. cereus*, as shown by multiple genomic DNA alignments. The *rpoB* gene has commonly been used for specific detection of *B. anthracis* (Qi et al. 2001; Ko et al. 2003; Oggioni et al. 2002). Here again a specific and reliable identification of *B. anthracis* can be affected by the presence of identical *rpoB* polymorphisms in the genome of *B. cereus* (Zasada et al. 2006). Indeed real-time assays based on this target can display delayed positive signals with *B. cereus*, presumably caused by mispriming (Klee et al. 2006a, b). The same observation applies to the *plcR* gene, a regulator of extracellular non-specific virulence genes in *B. thuringiensis* that is inactivated in *B. anthracis* due to a frameshift mutation (Agaisse et al. 1999; Mignot et al. 2001). According to current data, this frameshift mutation is considered to be *B. anthracis* specific. Nonetheless, delayed positive signals are recorded with some *B. cereus* isolates when using real-time assays and minor groove binding (MGB) probes (Easterday et al. 2005).

There is a wide spectrum of other DNA-based targets including the DNA replication gene *gyrA* (Hurtle et al. 2004), the *AC-390* gene (Cherif et al. 2002), the 16S rRNA gene, (Sacchi et al. 2002) and the *16S–23S rRNA* internal transcribed spacers (Daffonchio et al. 2000), the *vrrA* gene (Jackson et al. 1997), and the *SG-850* gene (Daffonchio et al. 1999). While *gyrA* was used for *B. anthracis* detection in real-time assay, most of the other target genes were used in end-point PCR assays requiring time-consuming post-PCR manipulations such as endonuclease restriction length polymorphism and subsequent gel electrophoresis. A genomic region, previously identified by subtractive hybridization, has recently been reported to be *B. anthracis* specific and was also used in a real-time PCR assay (Antwerpen et al. 2008). Although this genomic marker has so far not been found in non-*anthracis B. cereus*, the observation should now be confirmed on a larger panel of *Bacillus* of the *cereus* group. Altogether, and according to the current data, the unambiguous identification of *B. anthracis* using SNP-based discrimination and real-time PCR remains challenging. This has serious operational implications when one considers that first-line responders might be confronted with results of unclear significance, especially when delayed positive signals are recorded. In the field, it would then be extremely difficult to decide whether any delayed signal is indicative of mere presence of *B. cereus* or, alternatively, of low amounts of *B. anthracis* spores.

We have recently shown that the use of locked nucleic acid probes (LNAs) improves SNP-based identification of *B. anthracis* compared to MGB probes (Irenge et al. 2010). Furthermore, specificity of *B. anthracis* identification in environmental samples is improved by combining the detection of several chromosomal markers along with the identification of two virulence plasmids. This can either be achieved using multiplex real-time PCR (Wielinga et al. 2011) or by combining random or multiplex amplification with subsequent hybridization on a DNA chips (Burton et al. 2005; Burton et al. 2006). Whereas multiplex real-time PCR has already proved to be a suitable method in operational conditions (in the field), this is not yet the case for the DNA microarray scheme. The latter requires indeed sophisticated equipments for the hybridization and reading steps, and this method is prone to carry-over contamination if not performed in an adequate pre-and post-PCR setting.

Another promising nucleic acid amplification method for identification of microorganisms is the loop-mediated isothermal amplification (LAMP). LAMP relies on an isothermal auto-cycling strand displacement DNA synthesis using a DNA polymerase with high displacement activity and distinct sets of specific primers (Notomi et al. 2000). The reaction is carried out at a constant temperature range of 60–65°C for 1 h and only requires a heat blocker as equipment. This is a big advantage in terms of portability, especially in developing countries, where thermocyclers are not always available in laboratories. Accordingly, the technique has been used for simple and rapid detection of *B. anthracis*, with a limit of detection of 100 fg of bacterial DNA (Kurosaki et al. 2009). Recently, a LAMP using a disposable pocket warmer was developed for anthrax detection and was shown to yield a limit of detection limit of 1,000 copies of genomes of *B. anthracis* (Hatano et al. 2010). The use of a disposable pocket warmer frees the LAMP technique from the requirement to have electricity power supply. The method is very promising though its sensitivity needs to be improved in order to match real-time PCR.

Other molecular methods include multiple-locus sequence typing (MLST), multiple-locus variable-number tandem repeat analysis (MLVA), and next generation sequencing methods

(NGS). MLST is a method for characterising microbial isolates by means of sequencing internal fragments of housekeeping genes. The sequence data obtained for MLST can be used to construct dendograms in order to estimate genetic relatedness between isolates form the same species, for instance during outbreaks (Urwin and Maiden 2003). MLST has been recently introduced for the assessment of the relatedness between isolates belonging to the "*B. cereus*" group. The method has allowed clear-cut discrimination between *B. anthracis* isolates and other isolates of *Bacillus* of the *cereus* group (Helgason et al. 2004; Priest et al. 2004; Tourasse et al. 2006; Olsen et al. 2007); however, it is not sensitive enough to discriminate *B. anthracis* isolates. Recently, a new method was introduced using small repetitive elements appearing in a variable number and distributed among the genome of a given species. Accordingly this technique based on a variable number of tandem repeats was named multiple-locus variable-number tandem repeat analysis (Lindstedt 2005). In order to discriminate *B. anthracis* isolates, MLVA assesses several loci including chromosomal and plasmid loci. The technique allows valuable discrimination within *B. anthracis* isolates compared to MLST (Keim et al. 2000; Le Flèche et al. 2001; Valjevac et al. 2005; Lista et al. 2006). Nevertheless, these methods reveal to be insufficient to yield clear-cut discrimination of *B. anthracis* isolates within the same strain, as demonstrated by the Amerithrax investigation. This discrimination can only be achieved by whole genome sequence comparison between isolates (Rasko et al. 2011). For years, the use of this powerful investigative tool has been hampered by the cost and the delay for completing the whole process. The advent of next generation sequencing has dramatically changed the outcome by allowing rapid and high throughput sequence analysis.

NGS has been successfully used for discrimination of isolates from *B. anthracis* group and has helped discover novel strain-specific candidate SNPs previously overlooked by other typing methods (Cummings et al. 2010). NGS has now become a crucial tool in microbial forensics, especially in cases where intentional release is suspected as it was the case during the anthrax attacks in 2001. The Amerithrax investigation was successful because whole genome sequences of the *B. anthracis* isolates retrieved from the letter samples could be rapidly available, thus allowing analysis of sequences to characterize the isolates and link them to precise genotypes. This ultimately allowed investigators to close in on the origin of the attack (Rasko et al. 2011).

However and despite these spectacular achievements, typing methods (MLST, MLVA, and NGS) are not suitable for rapid identification of anthrax spores in environmental samples. These typing methods are indeed designed to work on isolated colonies, hence are unlikely to achieve relevant *B. anthracis* identification on field.

Future perspectives

Future development in identification of anthrax spores should focus on development of methods and technologies allowing more swiftness, more sensitivity, more sustainability, and more portability. A special focus must be on development of reagents free of the cold chain constraints. Conventional microbiological methods are clearly not suited for on field first-line responders because they require a heavy infrastructure only manageable in reach-back laboratories. Solid-support platforms using antibodies are easy to handle and are currently used in many countries, despite their low sensitivity (Lim et al. 2005) and specificity (Tengelsen et al. 2002). Another important issue is the cost related to antibodies production. Peptides such as bacteriophage lysins are an alternative to antibodies. Bacteriophage lysins are phage-encoded peptidoglycan hydrolases, which bind bacteria cell wall, and this binding brings about rapid lysis and death of the bacterial cell (Fujinami et al. 2007; Fenton et al. 2010). Peptide selection is carried out by the phage display technology (Turnbough 2003). This technology consists in creating a billion-clone library of recombinant phages expressing on their surface a large variety of peptides and proteins. These phage libraries are incubated with the antigen of interest and phages showing antigen affinity are then purified and propagated in *Escherichia coli* bacterial cells and then further purified using inexpensive technology (Petrenko and Vodyanoy 2003). The C-terminal region of the *B. anthracis*-specific γ-bacteriophage lysin (PlyG), especially the domain spanning from residues 156 to 233, shows a very high affinity for *B. anthracis* (Fujinami et al. 2007; Sainathrao et al. 2009; Kikkawa et al. 2007, 2008; Brigati et al. 2004; Williams et al. 2003), hence making it potentially interesting for *B. anthracis* spore detection (Rao et al. 2010). Consequently, PlyG-based probes have been developed for anthrax spore identification (Fujinami et al. 2007). These methods have a high potential for identification, but specificity issues still need to be carefully addressed because this peptide was also demonstrated to bind to *B. cereus* spores (Sainathrao et al. 2009).

Another alternative for affinity-based identification of *B. anthracis* consists in using aptamers. Aptamers are indeed short single-stranded nucleic acids (RNA, ssDNA, and modified RNA) that can specifically recognize their own target on the basis of its unique tridimensional structure. Aptamers are usually considered to be oligonucleotides analogs to antibodies (Faulhammer et al. 2004; Huang et al. 2007; Jeong et al. 2009; Lim et al. 2010). The purification of these nucleic acid sequences with unique properties is achieved through iterative cycles of in vitro selection from a random pool of sequences with a method called systematic evolution of ligands by exponential enrichment (Ellington and Szostak 1990; Tuerk and Gold 1990; Stoltenburg et al. 2007; Dua et al. 2011). Aptamers are attracting attention in the field of clinical diagnosis because they are claimed to have many advantages

compared to antibodies (i.e., high affinity, small size, little immunogenicity, stable structures, and easy production compared to antibodies). However, despite these alleged qualities, there has been up to now no real breakthrough in the use of aptamers as a typing method. Accordingly, development of new identification methods is very slow, and there is no indication of whether aptamer-based assays will soon be available for *B. anthracis* identification in environmental samples. Except for an anthrax spore-specific electro-chemiluminescent assay (Bruno and Kiel 1999), and a protecting antigen-specific ELISA assay (Choi et al. 2011), no validated data have so far been reported on the use of aptamers for anthrax spore identification. Emerging affinity-based techniques aim at improving sensitivity of *B. anthracis* detection. These include techniques such electroluminescence (ECL), immuno-magnetic ECL, aptamer-magnetic bead ECL, and biosensors (Herzog et al. 2009). A biosensor detects chemical and biological compounds present in the environment through its specific biological recognition element whose property changes upon target binding. This change is converted into a signal that can be conditioned and quantified (Nayak et al. 2009). Various sensor devices have been reported for *B. anthracis* detection. They include sensor devices such as evanescent fiber-optic biosensors (Lim 2003; Tims and Lim 2004), ECL assay (Bruno and Yu 1996; Yu 1998), quartz crystal microbalance (Hao et al. 2009), and cantilever-based sensors (Campbell and Mutharasan 2006; Davila et al. 2007). The bioreceptor can be nucleic acid probes that specifically bind *B. anthracis* DNA. In this type of assay, hybridization of the DNA target on its corresponding probe results both in an increased mass and a decreased resonance frequency of the quartz microbalance biosensor (Hao et al. 2011). An interesting review on the use of biosensors for identification of microorganisms has been recently published (Kim and Yoon 2010).

The real-time PCR has improved assays sensitivity of to unexpected levels while bringing automation and improving intra and interassay reproducibility. Quantification of genomes and/or plasmids yields valuable information on microorganisms load in the sample. However, specificity is still an issue. The selection of novel molecular targets and improvement of SNP-based discrimination (through development of new probes such as LNA) are set to improve specificity of real-time PCR assays. Equipment is still big and too reliant on power supply to be easily moved on the scene. The advent of LAMP is a huge breakthrough in this respect though its sensibility needs to be improved in order to compare favorably with real-time PCR.

Conclusion

The intentional release of *B. anthracis* spores in the fall of 2001 made public health officials acutely aware of the importance of rapidly and accurately detecting both natural and intentional release of deadly pathogens. Since then, the scientific community has strived to develop efficient methods for rapidly detecting and identifying biowarfare and biothreats agents, among which *B. anthracis* ranks high. Despite these commendable efforts, detection of *B. anthracis* in environmental samples remains still challenging in terms of sensibility and specificity. This is particularly true for affinity-based methods, which belong to the few methods that first-line responders could easily rely on for detecting and identifying *B. anthracis* spores. Considering the potential of DNA-based assay, nucleic acid-based detection systems are expected to substantially improve the detection of *B. anthracis* spores in environmental samples. An appropriate combination of the best DNA targets, the development of more specific probes, as we reported it with LNA probes, and the advent of portable real-time PCR machines and the ongoing development of LAMP devices are set to improve molecular in field-identification of anthrax spores. A specific and sensitive identification may however require a multi-dimensional approach given that current *B. anthracis* detection methods are designed to detect only a single dimension at the time: nucleic acids, antigens, toxins, or whole bacterial cells. Due to their genuine limitations, none of those limited assays can fulfill this task on their own, but a combination thereof should significantly improve on-site detection of *B. anthracis* spores, hence significantly reducing the false alarm rate recorded with these difficult samples. It is of note that the discovery of closely related *B. cereus* able to cause an *anthrax*-like disease in animals should not be forgotten when designing methods for a rapid detection of anthrax disease and for identification of its causative agent.

Acknowledgments This work was supported by the Department Management of Scientific & Technological Research of Defence (IRSD-RSTD; Royal High Institute for Defence) supporting research and development (grants MED-05 and MED-08) and by the Walloon Region (grant 616313).

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