# Original Article

# Real-Time PCR Identification of Unique *Bacillus anthracis* Sequences

(*Bacillus anthracis* / real-time PCR / identification / *BA5345* / *pag* and *cap* genes)

P. CIEŚLIK[1], J. KNAP[2], M. KOŁODZIEJ[1], T. MIRSKI[1], J. JONIEC[1], G. GRANIAK[1], D. ŻAKOWSKA[1], I. WINNICKA[3], A. BIELAWSKA-DRÓZD[1]

[1]Biological Threats Identification and Countermeasure Centre of the General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Puławy, Poland
[2]Warsaw Medical University, Second Faculty of Medicine, Department of Epidemiology, Warsaw, Poland
[3]General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Epidemiology Department, Warsaw, Poland

**Abstract. *Bacillus anthracis* is a spore-forming, Gram-positive microorganism. It is a causative agent of anthrax, a highly infectious disease. It belongs to the "*Bacillus cereus* group", which contains other closely related species, including *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus weihenstephanensis,* and *Bacillus pseudomycoides. B. anthracis* naturally occurs in soil environments. The *BA5345* genetic marker was used for highly specific detection of *B. anthracis* with TaqMan probes. The detection limit of a real-time PCR assay was estimated at the level of 16.9 copies ($CI_{95\%}$ - 37.4 to 37.86, SD = 0.2; SE = 0.118). Oligonucleotides designed for the targeted sequences (within the tested locus) revealed 100 % homology to *B. anthracis* strain reference sequences deposited in the database (NCBI) and high specificity to all tested *B. anthracis* strains. Additional *in silico* analysis of plasmid markers *pag* and *cap* genes with *B. anthracis* strains included in the database was carried out. Our study clearly indicates that the *BA5345* marker can be used with success as a chromosomal marker in routine identification of *B. anthracis*; moreover, detection of plasmid markers indicates virulence of the examined strains.**

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Corresponding author: Agata Bielawska-Drózd, Biological Threats Identification and Countermeasure Centre of the General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Lubelska 2 St., 24-100 Puławy, Poland. E-mail: pcieslik@wihe.pulawy.pl

Abbreviations: CI – confidence interval, EF – oedema factor, LF – lethal factor, PA – protective antigen, SD – standard deviation, SE – standard error, SNP – single-nucleotide polymorphism.

## Introduction

*Bacillus anthracis* is a causative agent of anthrax, an infectious disease dangerous or even fatal for humans and animals. It is a representative of the so called "*Bacillus cereus* group" described in 1980 as a distinct taxonomic group within the genus *Bacillus*. This group also includes *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*. The bacteria are genetically similar to the extent of being almost indistinguishable; the high degree of similarity is of importance particularly in the classification of *B. thuringiensis*, *B. cereus*, and *B. anthracis* due to the possibility of horizontal gene transfer between the species occurring in the soil under favourable conditions, i.e. adequate moisture, temperature, pH (alkaline), and the presence of various nutrients and ions, especially $Ca^{2+}$ and $Mn^{2+}$. In the case of "*Bacillus cereus* group", the bacterial life cycle takes place entirely in the soil (Van Nes, 1971).

Gene transfer can be performed by moving mobile elements present in pathogenicity islands, i.e. shifting entire plasmids, by transduction involving the CP-51 phage, or by transposons, such as the transfer of Tn 4430-containing plasmid pXO12 from *B. thuringiensis* (Green et al., 1989; de la Cruz and Davies, 2000; Helgason et al., 2000; Ellerbrok et al., 2002; Pepper and Gentry, 2002). The existence of so-called transitional strains of *Bacillus sp. Ba813*$^{+}$ (Ramisse et al., 1999) confirms genetic changes in soil-dwelling bacteria. The phenomenon of gene transfer may cause diagnostic difficulties. Literature presents cases of *B. cereus* acquiring the virulence determinants typical of *B. anthracis* (Hoffmaster et al., 2004), as well as the occurrence of *B. anthracis* losing pXO2 and pXO1 plasmids (de la Cruz and Davies, 2000; Pannucci et al., 2002). A case of isolation of *B. cereus* from a patient with acute pneumonia symptoms has also been reported. The isolated G9241 strain of *B. cereus* carried the pBCXO1 plasmid

that displayed 99.6 % similarity to the pXO1 *B. anthracis* plasmid. Although it did not possess the pXO2 plasmid, biological tests revealed 100 % mortality (Hoffmaster et al., 2004).

In recent years, there have been attempts to adopt a variety of molecular biology techniques based on genotyping in identification and differentiation of *B. anthracis*: Multilocus Sequence Typing (MLST) (Tourasse et al., 2006; Olsen et al., 2007), Multi Locus VNTR Analysis (MLVA) (Keim et al., 2000; Lista et al., 2006) and real-time PCR (Qi et al., 2001; Drago et al., 2002; Ellerbrok et al., 2002; Hoffmaster et al., 2002; Bode et al., 2004; Antwerpen et al., 2008).

Molecular identification based only on *B. anthracis* plasmid genes has become insufficient (Ellerbrok et al., 2002). It is also necessary to confirm the presence of a specific chromosome sequence. Many previous studies have been focused on chromosomal markers, such as *Ba813* (Ramisse et al., 1999; Brumlik et al., 2004), *SG-850* (Daffonchio et al., 1999; Ticknor et al., 2001), *vrrA* (Jackson et al., 1997; Keim et al., 2000; Kim et al., 2002; Pannucci et al., 2002), *rpoB* (Qi et al., 2001; Ellerbrok et al., 2002), *gyrA* (Drago et al., 2002; Hurtle et al., 2004), *Ba5510* (Olsen et al., 2007), and *plcR* (Easterday et al., 2005). However, later results indicate that these sequences can also be present in other members of the "*Bacillus cereus* group" (Ellerbrok et al., 2002; Bode et al., 2004). Among the presently examined chromosomal markers, the most promising are sequences of genes encoding hypothetical proteins: *BA2686* (Bode et al., 2004) and *BA5345* (Antwerpen et al., 2008).

To determine the virulence of *B. anthracis* isolates, *pag* and *cap* genes should be detected. The genes encoding the virulence factors of *B. anthracis* are located on two plasmids: pXO1 (185 kbp) and pXO2 (95 kbp). The pXO1 plasmid contains: the *pag* gene encoding a protective antigen (PA) (85 kDa), the *cya* gene encoding the oedema factor (EF) (89 kDa), and the *lef* gene encoding the lethal factor (LF) (83 kDa). The second capsule-encoding plasmid, pXO2, contains the genes *capA, capB* and *capC*. Fully virulent strains of *B. anthracis* carry two plasmids, and the loss of one or both of them ($pXO1^-$, $pXO2^+$; $pXO1^+$, $pXO2^-$ or $pXO1^-$, $pXO2^-$) results in the lack of their virulence.

Due to the possibility of horizontal gene transfer, rapid and accurate detection of *B. anthracis* should be implemented.

The aim of this study was to discover the unique chromosomal and plasmid sequences by testing *in silico* (Primer BLAST, *in silico* PCR), designing molecular probes (TaqMan probe), and testing them with the representatives of the "*Bacillus cereus* group" and distantly related species using real-time PCR. The emphasis of the study was placed on the testing of chromosomal sequences, while reaction specificity of the plasmid *pag* and *cap* genes was tested in the second stage of the study using the same methodology.

## Material and Methods

For all strains of *B. anthracis* deposited in the BTICC (Biological Threat Identification and Countermeasure Centre, Puławy, Poland) collection, the presence of the unique chromosomal sequence *BA5345* (genomic location in *B. anthracis* Ames: 4845077 – 4845189 nt) was confirmed. Real-time PCR with newly designed primers BAR: 5' – CCTATAGAAGCGGATTTGTC – 3', BAF: 5' – CCATTGATTCATCACCCAAG – 3' and TaqMan probe BAS: 5' – FAM – TACACCCACAGCGGCTAAAGAG – BHQ1 – 3' was carried out. The nucleotide sequence *BA5345* can effectively distinguish closely related species in the "*Bacillus cereus* group"; it may therefore be a useful tool for rapid and precise identification of *B. anthracis* by real-time PCR in various samples. To determine the virulence of *B. anthracis* it is necessary to use sets of oligonucleotides designed for the identification of genes encoding the anthrax toxin and capsule. For this purpose the following sets of primers and probes were used: for the *pag* gene: Fpag: 5'-GAATCAAGTTCCCAGGGGTTACTAGG – 3', Rpag: 5' – CATGATTATCAGCGGAAGTAGC – 3' and the probe: Spag: 5' – FAM – GCACCCATGGTGGTTACCTCTTCTACT – BHQ1 – 3', for the *cap* gene: primers Fcap 5' – TCGTACTGAGCAGTTTGCTA – 3', Rcap: 5' – CGTTGGAATATCTCCTT – 3' and the probe – Scap: 5'–FAM–TGCGATTGGAGAAACGACTGCAC – BHQ1 – 3'. The oligonucleotides were synthesized at Genomed (Warsaw, Poland).

Primers and probes for specific marker gene sequences were designed using the LightCycler Probe Design 2.0 program (Roche, Mannheim, Germany). In order to improve and verify the parameters of primers and probes, the Oligo Analyzer 1.0 and Fixed Oligos software was used. The designed nucleotide sequences were subsequently analysed by *in silico* PCR for the specificity of sequences deposited in the NCBI database. The obtained amplicons were then compared using the ClustalW2 algorithm in order to check the homology of the designed primers and probes with marker sequences. In addition, a comparative analysis of primers and amplicons using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also carried out. The amplification products were found to be different from the other members of the "*Bacillus cereus* group", and there was no cross-reactivity of the designed oligonucleotides against closely related bacteria.

Isolation of genetic material was performed using the automatic instrument MagNA Pure (Roche) with a DNA Nucleic Acid Isolation Kit I (Roche), according to the manufacturer's recommendations. The concentration of obtained DNA was approximately 10 ng/µl.

Real-time PCR reactions using TaqMan probes for all three designated sequences (*BA5345, pag, cap*) were carried out with the following mixture: 0.5 µM of primers (final concentration), 0.15 µM (final concentration) of probe, LightCycler TaqMan Master kit (Roche) 4 µl, and 3 µl of 0.1 – 1 ng/µl DNA solution. The PCR cy-

cling conditions for all runs in LightCycler 2.0 (Roche) were:

– for the *BA5345* gene with TaqMan Probes: initial denaturation 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min,
– for the *pag* gene with TaqMan Probes: initial denaturation 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min,
– for the *cap* gene with TaqMan Probes: initial denaturation 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 1 min.

The study was carried out with five *B. anthracis* isolates (*B. anthracis 1583*, *B. anthracis 1584*, *B. anthracis 211*, *B. anthracis 1153*, *B. anthracis* $34F_2$-vaccine strain) and a panel of 60 control isolates belonging to the closely related "*Bacillus cereus* group", as well as other distantly related species. All isolates were from the ATCC (American Type Culture Collection, Manassas, VA, from the collection of the Institute of Molecular Biology and Medicine (IMBM) at the University of Scranton, PA), and from the collection of the Department of Medical Microbiology, Medical University of Lublin, Poland. Among the study group the following were highlighted: *B. cereus* ATCC 10876, *B. cereus* ATCC 23261, *B. cereus* ATCC 14579T-27, *B. cereus* ATCC 13472, *B. cereus* ATCC 19637, *B. cereus* F17202-30, *B. cereus* UW 85-28, *B. cereus* T7-101, *B. cereus* F16959-24, *B. cereus* F17285S-29, *B. cereus* UM1, *B. cereus* UM2, *B. thuringiensis* ATCC 10792T, *B. thuringiensis* ATCC 33676, *B. thuringiensis* ATCC 33679, *B. thuringiensis* ATCC 35646, *B. thuringiensis* T07-146 *B. thuringiensis* #33, *B. thuringiensis* #35, *B. thuringiensis* #37, *B. thuringiensis* T7-030, *B. thuringiensis* T07-148, *B. thuringiensis* T07-202, *B. thuringiensis* T07-005, *B. thuringiensis* T07-153, *B. thuringiensis* T07-001, *B. thuringiensis* T07-019, *B. thuringiensis* T7-113, *B. thuringiensis* T7-128, *B. thuringiensis* -151, *B. thuringiensis* T7-019, *Bacillus* spp. *Ba813*$^+$#25(97-24), *Bacillus* spp. *Ba813*$^+$#28(III), spp. *Ba813*$^+$#11(9594/3), *Bacillus* spp. *Ba813*$^+$#17(094), *Bacillus* spp. *Ba813*$^+$#21(TII97-3), *Bacillus* spp. *Ba813*$^+$#13(PC1), *Bacillus* spp. *Ba813*$^+$#16(PJ572), *Bacillus* spp. *Ba813*$^+$#15(11614-2), *Bacillus* spp. *Ba813*$^+$#7 (II/3), *Bacillus* spp. *Ba813*$^+$#31, *Bacillus* spp. *Ba813*$^+$ #S8553/2, *Bacillus* spp. *Ba813*$^+$#23(III-SL), *Bacillus* spp. *Ba813*$^+$#19(T579-77), *Bacillus* spp. *Ba813*$^+$#12 (S8553/2), *Bacillus* spp. *Ba813*$^+$#3403, *Bacillus* spp. *Ba813*$^+$#30(1B), *Bacillus* spp. *Ba813*$^+$#22 Bu-1M, *Bacillus* spp. *Ba813*$^+$#6(I/2), *Bacillus* spp. *Ba813*$^+$#14, *Bacillus* spp. *Ba813*$^+$#22(Br-13), *Bacillus* spp. *Ba813*$^+$ #18(T297-76), *Bacillus* spp. *Ba813*$^+$#24(IIIB5), *Bacillus* spp. *Ba813*$^+$#29 IV, *Bacillus* spp. *Ba813*$^+$#28(3), *Bacillus* spp. *Ba813*$^+$#25(97-27), *B. subtilis* ATCC 6633, *B. subtilis* UM1, *B. subtilis* UM2, *B. megaterium* UM1, *B. megaterium* UM2. Statistical calculations were performed using Student's *t*-test.

## Results

An *in silico* comparison of the *BA5345* sequence with the selected reference *B. anthracis* strain genomes (*B. anthracis* Ames, *B. anthracis* Ames Ancestor, *B. anthracis* CDC 684*, B. anthracis* A0248, *B. anthracis* Sterne, *B. anthracis* H9041) deposited in the NCBI database was also performed. An amplification product of 132 bp was obtained in a real-time PCR assay with primers and hydrolysing probes designed for the *BA5345* sequence. Additionally, in the presence of appropriate plasmid markers, the products of 223 bp (Fpag/Rpag) and 119 bp (Fcap/Rcap) were obtained (data not shown). As a result, 100 % identity between the tested strains was found.

Additionally, available genomes of *B. anthracis* were compared using the PSAT algorithm (Procaryotic Sequence Homology Analysis Tool) (Fong et al., 2008), with genomes of *B. anthracis* reference strains deposited in the database in terms of homological sequences. As a result, it was found that common, specific homological sequences were present under different names (*BA5345*, *GBAA5345, BAMEG5398, BAA5375, BAS4966, H9041_5097* (Fig. 1) in different strains.

In addition, the BLAST program was used to compare the amplicon for the *BA5345* sequence with BAF and BAR primers in "whole genome shotgun reads" and "nucleotide collection" databases (data not shown).

Results of the testing of chromosomal and plasmid markers revealed no cross-reactivity between *B. anthracis* strains and closely and distantly related species.

The detection limit of a real-time PCR assay using a TaqMan probe (BAS) specific to the *BA5345* sequence was determined using the *B. anthracis* $34F_2$ strain according to the Létant scheme (Létant et al., 2011). Concentration of genomic DNA was measured by the spectrophotometric method using the NanoDrop 2000 instrument (Thermo Scientific, Schwerte, Germany). Subsequently, a series of ten-fold serial dilutions in the range 10 ng/µl to 1 fg/µl was prepared. The mean values of three cycle threshold ($C_t$) values for each dilution were compared to DNA concentrations with the correlation of determination $R^2 = 0.9995$. In our examination, 95 % confidence interval ($CI_{95\%}$), standard deviation (SD) and standard error (SE) were calculated. The genome copy number was established using the "Calculator for determining the number of copies of a template" (http://cels.uri.edu/gsc/cndna.html). The limit of the real-time PCR assay was estimated at the level of 16.8 copies ($C_t$ = 37,45; $CI_{95\%}$ – 37.4 to 37.86; SD = 0.2; SE = 0.118).

## Discussion

Diagnostics of *B. anthracis* is concentrated on detection of specific sets of genetic markers, including chromosomal markers such as *rpoB* (Qi et al., 2001; Ellerbrok et al., 2002), *BA2686* (Bode et al., 2004)*, BA5345* (Antwerpen et al., 2008), *pag* gene (located on the pXO1 plasmid) and *cap* gene (located on the pXO2 plasmid).

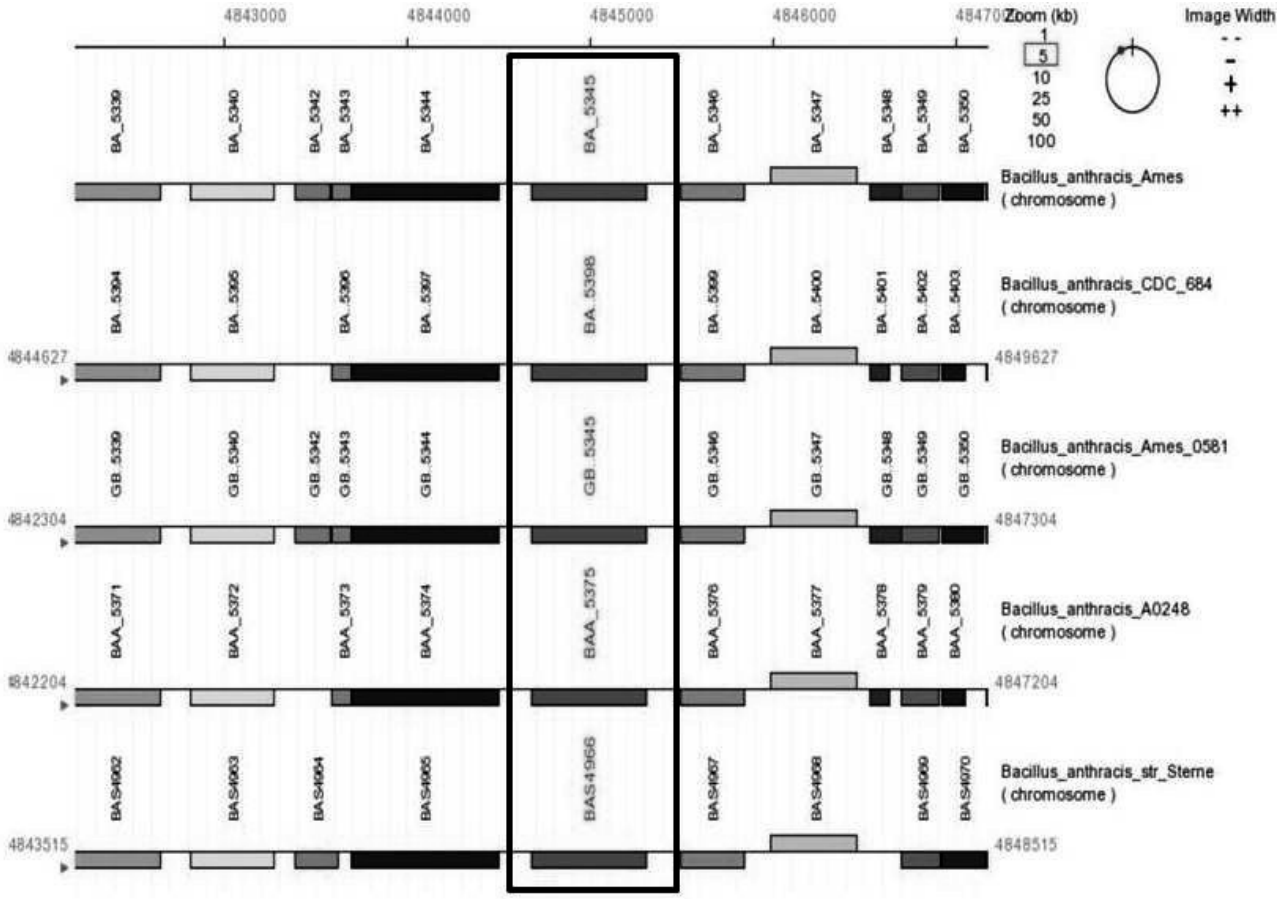


*Fig 1.* Genomic location of *BA5345* homological sequences in other strains of *B. anthracis*

The results of our research revealed that oligonucleotides designed for the *BA5345* sequence (genomic location in *B. anthracis* Ames: 4845077 – 4845208 nt) are highly specific for *B. anthracis*. The identity of the examined *BA5345* sequence and the obtained amplicon were tested using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) in relation to the reference strain sequence within the tested locus. Due to its high homology the sequence may be useful as a marker for the identification of *B. anthracis*. Furthermore, the *BA5345* targeted sequence presented specificity to all examined *B. anthracis* strains (*B. anthracis 1583*, *1584*, *211*, *1153* and *34F*$_2$) in relation to 60 closely and distantly related control species (P = 0.0441). In addition, no amplification products were detected in the *in silico* analysis of primer pair BAF and BAR for the "*Bacillus cereus* group" members, which are closely related and, according to the literature, may cause clinical symptoms similar to anthrax. The following strains were included in the analysis: *B. cereus* 03BB108, *B. cereus* NVH0597-99, *B. cereus* E33L, *B. cereus* G9241, *B. thuringiensis* Al Hakam, and *B. thuringiensis* 97-27 (Antwerpen et al., 2008).

Other authors (Bode et al., 2004; Antwerpen et al., 2008) also designed a specific real-time PCR method with genes *BA2686* and *BA5345* encoding hypothetical proteins. However, the targeted sequences belonged to different *B. anthracis* gene loci than in this study.

Among 42 *B. anthracis* and 53 *Bacillus* species, specific products were observed only for *B. anthracis*. Additionally, the authors found the detection limits to be comparable with Bode et al. (2004). Antwerpen et al. (2008) tested oligonucleotides designed for the *BA5345* gene (genomic location in *B. anthracis* Ames: 4846162 – 4846257 nt) against 92 *B. anthracis* isolates and 236 other strains belonging to the *Bacillus* genus and did not obtain any non-specific products. The detection limit was 12.7 genome copies, which is comparable with the results of this study. Conversely, Ellerbrok et al. (2002) tested the *rpoB* gene and self-designed primers and probe, and received non-specific products in the case of four isolates of *B. cereus* and one isolate of *B. megaterium.* Their results may be explained by a higher variability of the *rpoB* gene among the *Bacillus* genus.

In the literature, several strains of non-*B. anthracis* species containing virulence determinants of anthrax have been described. This phenomenon may be due to horizontal gene transfer, by which genes or plasmids can be transferred between closely related species.

Oligonucleotides designed for the *pag* (pXO1) and *cap* (pXO2) genes were analysed *in silico*. The *pag* gene analysis showed the amplification product for all *B. anthracis* reference strains, and also for the strains of *B. cereus* 03BB102 (Létant et al., 2011) and *B. cereus* G9241 (accession number AAEK01000020.1), where the presence of the *pag* gene had previously been de-

scribed (Hoffmaster et al., 2004, 2006). The sequence identity of the amplicon for *B. cereus* M1550 (Accession number ACMA01000109.1) was detected in the section between 86–223 nucleotides (138 bp, 100 % identity). Regarding the *cap* gene (amplicon size: 119 bp), the *in silico* analysis of the deposited genomes of *B. anthracis* strains and other *Bacillus spp*. showed the presence of the *cap* gene in the *B. cereus* 03BB108 strain (Létant et al., 2011) (accession number ABDM02000063.1). Widespread analysis of the sequence revealed nine single-nucleotide polymorphisms (SNPs) in the compared area. Moreover, the *in silico* analysis showed 96 % sequence homology with the plasmid p03BB102_179 (accession number CP001406) of *B. cereus* 03BB102 (range: 88111-87998 nt) and, interestingly, comparison of the full-length *cap* gene of *B. anthracis* CDC684 and *B. cereus* 03BB102 showed 96 % similarity (45 SNPs were found). Furthermore, the presence of the amplified *cap* gene fragment was also detected in *B. circulans* CBD118 (accession number DQ517347) and *Bacillus* spp. CBD119 (accession number DQ517348), with 100 % identity.

As this study revealed, purpose-made molecular tools show high specificity for identification of *B. anthracis* and may be successfully used in routine diagnostic tests. In order to achieve full identification of *B. anthracis*, it seems advisable first to detect the chromosomal sequence and in the next stage, to identify *pag* and *cap* genes located on plasmids for the determination of virulence of the tested isolates.

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