Evaluation of real-time PCR based on SYBR Green I fluorescent dye for detection of *Bacillus anthracis* strains in biological samples

Agnieszka Kędrak-Jabłońska, Sylwia Budniak, Anna Szczawińska,
Monika Reksa, Marek Krupa, Krzysztof Szulowski

Department of Microbiology,
National Veterinary Research Institute, 24-100 Pulawy, Poland
akedrak@piwet.pulawy.pl

Abstract

Introduction: The aim of the study was the application and evaluation of real-time PCRs based on the fluorescence of SYBR Green I intercalating dye for the detection of three *Bacillus anthracis* genes in contaminated liver and blood samples. The goals for detection were *rpoB* gene as a chromosomal marker, *pag* gene located on plasmid pXO1, and *capC* gene located on plasmid pXO2. **Material and Methods:** Five *B. anthracis* strains were used for the experiments. Additionally, single strains of other species of the genus *Bacillus*, *i.e. B. cereus*, *B. brevis*, *B. subtilis*, and *B. megaterium*, and strains of six other species were used for evaluation of the specificity of the tests. Three SYBR Green I real-time PCRs were conducted allowing confirmation of *B. anthracis* in the biological samples. **Results:** The observation of amplification curves in real-time PCRs enabled the detection of the chromosomally encoded *rpoB* gene, *pag* gene, and *capC* gene of *B. anthracis*. The specificity of the tests was confirmed by estimation of the melting temperature of the PCR products. The sensitivity and linearity of the reactions were determined using regression coefficients. Strains of other microbial species did not reveal real-time PCR products. **Conclusion:** All real-time PCRs for the detection of *B. anthracis* in biological samples demonstrated a significant sensitivity and high specificity.

Keywords: *Bacillus anthracis*, real-time PCR, SYBR Green I.

Introduction

Anthrax is an infectious disease caused by *Bacillus anthracis* which usually takes the form of septicaemia. The characteristic lesions include an enlarged spleen and poorly clotted blood. The disease is most common in herbivorous animals, and less frequent in omnivorous and carnivorous animals and humans (3, 28).

In cattle, the course of the disease is often acute and leads to death after one or two days. In several cases seemingly healthy animals infected with anthrax suddenly fall and convulse on the pasture. Anthrax also demonstrates an acute or peracute course in sheep and goats. These animals show nervous system symptoms during this short-term illness. Horses infected with anthrax present symptoms of colic resulting from the disease processes developing in the gastrointestinal tract. In pigs, as in the animal species mentioned above, the disease follows the course of septicaemia or is limited to lesions in the larynx and trachea. *B. anthracis* is also pathogenic for carnivores and causes inflammation of the stomach, intestines, and throat, and gum ulceration. Anthrax has also been reported in domestic fowl although these birds are far less sensitive to it than herbivorous mammals (3, 27).

In humans anthrax may demonstrate one of three forms depending on the route of infection. The cutaneous form is associated with skin damage and infection including a blister with a serosanguineous exudate that dries into a black gangrenous scab. The pulmonary form develops as a result of inhaling anthrax spores and proceeds in the form of bronchopneumonia usually leading to death. Contaminated food or water may result in the gastrointestinal form with the symptoms of bloody

diarrhoea, vomiting, cyanosis, vascular system disorders, and death (6, 27, 28).

The basis for *B. anthracis* identification is bacterial culture. However, the entailed laboratory diagnostics, based on the assessment of colony morphology, physiological and biochemical properties, are time-consuming and require specialised tests. The identification of *B. anthracis* is complicated because the pathogen is closely related to several species of environmental bacilli including *B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis,* and *B. weihenstephanesis* (4, 15, 23). *B. cereus* species show a similar cellular structure and physiology, but they differ from *B. anthracis* in pathogenicity. The high genetic similarity of these species may pose identification problems when conventional diagnostic methods are used (10, 12, 24). Molecular biological methods are developed on the elaboration of the specific and sensitive PCR and real-time PCRs that are used to identify *B. anthracis*.

The aim of the study was the application and evaluation of a real-time PCR based on the fluorescence of SYBR Green I dye for detection of the *rpoB* gene as a chromosomal marker and genes located on the pXO1 (*pag* gene) and pXO2 (*capC* gene) plasmids of *B. anthracis* strains in contaminated biological samples (5, 7, 25).

Material and Methods

Bacterial strains. The study involved five *B. anthracis* strains (B.a. 1/47, B.a. 7/51, B.a. 13/93, B.a. 15/93, and B.a. 16/96). Strains of other species of the genus *Bacillus, i.e.* single strains of *B. cereus* ATCC 11778, *B. brevis* 1114, *B. subtilis* ATCC 6633, and *B. megaterium* 1534 were also included in the experiment. To assess the specificity of the tests, the experiments involved strains of other species, including *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644, *Pasteurella multocida* M1404, *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, and *Klebsiella pneumoniae* ATCC 13883. The strain of *P. multocida* M1404 originated from National Animal Disease Centre, Ames, USA and other strains from the NVRI, Department of Microbiology's own collection.

Isolation of DNA. Each strain was streaked on TSB medium (bioMérieux, France) and incubated for 18 h at 37ºC. Bacterial cultures of the examined strains were used to contaminate bovine liver and blood. The samples were preliminarily incubated with mutanolysin or lysozyme, and then kits from A&A Biotechnology (Poland) and Qiagen (USA) were used, respectively. Isolation of DNA from liver samples was performed using a DNA Genomic Mini (A&A Biotechnology, Poland) and a DNeasy Blood and Tissue Kit (Qiagen, USA). In the case of blood samples, DNA was isolated with the use of a Genomic Blood Mini and a DNeasy Blood and Tissue Kit.

The sensitivity and linearity of real-time PCRs for detection of *B. anthracis* were determined by the 10-fold dilution technique. The initial concentration of strain B.a. 1/47 was about 3.5×10^4 CFU/reaction.

SYBR Green I real-time PCR. A QuantiTect SYBR Green PCR Kit (Qiagen, USA) was used. The reaction was performed in a 25 μL mixture containing the appropriate concentration of each primer and 5 μL of DNA. Sequences of primers for amplification were based on the literature data in accordance with Ellerbrok *et al.* (7). The primers were synthesised in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (IBB PAN), in Poland. The characteristics of the real-time PCR primers are shown in Table 1. The effect of primer concentrations (0.1 mM, 0.25 mM, 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM) was evaluated to optimise the reaction. Reactions were run on a Rotor-Gene Q cycler (Qiagen, USA) and Rotor-Gene Q Series software version 2.0.2 with the following programme: 10 min at 95°C and 40 cycles of 30 s at 95°C, 30 s at 55°C, and 90 s at 72°C. To confirm amplification specificity, the melting temperature of PCR products was determined by gradually raising the reaction mixture temperature from 55°C to 95°C and continuously measuring fluorescence.

**Table 1.** Characteristics of the primers for real-time PCRs

Primers		Sequence (5'–3')	Concentration
rpoB gene	rpoB-F	CCACCAACGTAGAAAATGCC	1.0 μM
	rpoB-R	AAATTTCACCAGTTTCTGGATCT	
pag gene	PA-F	CGGATCAAGTATATGGGAATATAGCAA	0.1 μM
	PA-R	CCGGTTTAGTCGTTTCTAATGGAT	
capC gene	Cap-F	ACGTATGGTGTTTCAAGATTCATG	1.5 μM
	Cap-R	ATTTTCGTCTCATTCTACCTCACC	

Results

The first stage of experiment focused on the influence of different primer concentrations. It was found that in the case of real-time PCR for detecting *rpoB* gene with the rpoB-F and rpoB-R primers, their optimal concentration was 1.0 μM. For detection of *pag* gene with the PA-F and PA-R primers, the optimal concentration was 0.1 μM, whereas for detecting *capC* gene with the Cap-F and Cap-R primers it was 1.5 μM.

In the next stage, a SYBR Green I real-time PCR was performed for detecting the *rpoB* gene with template DNA obtained from the liver samples contaminated experimentally with five *B. anthracis*

strains. Ct values ranged from 25.59 to 30.97 for the Genomic Mini (A&A Biotechnology, Poland) and from 14.56 to 25.34 for the DNeasy Blood and Tissue Kit (Qiagen, USA). When the Genomic Blood Mini and DNeasy Blood and Tissue Kit isolated DNA from contaminated blood samples, Ct values from 21.29 to 26.89 and from 11.65 to 25.62 were obtained, respectively. In all five examined strains the *rpoB* gene was detected. The specificity of amplification was confirmed by determination of the PCR product melting temperature, which was found to be about 78°C. The sensitivity of the real-time PCR was also determined. Isolating DNA from contaminated liver samples using the Genomic Mini, the sensitivity was 3.5×10^0 CFU/reaction, and using the DNeasy Blood and Tissue Kit it was 3.5×10^1 CFU/reaction. The sensitivity was established at 3.5×10^1 CFU/ reaction for isolation of DNA from contaminated blood samples, with the use of the kits from both manufacturers (Fig. 1). In the case of template DNA obtained from experimentally contaminated biological samples, with the use of the kits from A&A Biotechnology (Poland) and Qiagen (USA), a high regression coefficient 0.99 was demonstrated.

a)

b)
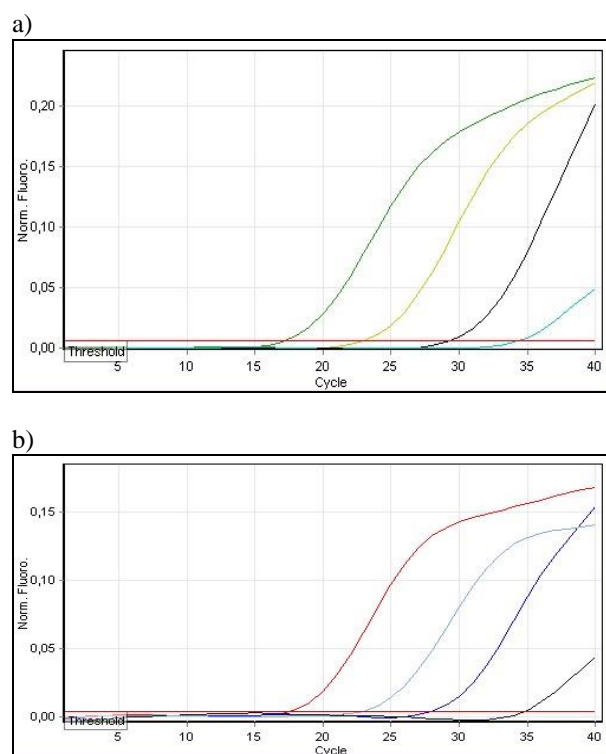
Fig. 1. Determination of the sensitivity of SYBR Green I real-time PCR with rpoB-F/R primers for the detection of *B. anthracis* strain 1/47 in liver (a) and blood (b) samples using a DNeasy Blood and Tissue Kit for DNA isolation

Next, real-time PCR detection of the *pag* gene located on plasmid pXO1 was carried out. For template DNA isolated from contaminated liver samples, Ct values fell within of 17.72 to 22.90 using the Genomic Mini and 18.24 to 22.83 for the DNeasy Blood and

Tissue Kit. Ct values ranged from 15.12 to 20.69 and from 15.85 to 20.78 for DNA isolated from contaminated blood samples with the use of the Genomic Blood Mini and DNeasy Blood and Tissue Kit, respectively. One of the five examined *B. anthracis* strains (B.a. 7/51) did not show any real-time PCR product, which indicates the absence of the *pag* gene. The melting temperature of the reaction products was found to be about 79°C. The sensitivity of real-time PCR with the use of PA-F and PA-R primers was 3.5×10^1 CFU/reaction for DNA obtained from contaminated liver samples using both kits. Regarding DNA isolated from contaminated blood samples using the Genomic Blood Mini the sensitivity was 3.5×10^1 CFU/reaction and with the use of the DNeasy Blood and Tissue Kit was 3.5×10^0 CFU/reaction (Fig. 2). A high regression coefficient 0.99 was also shown.

a)

b)
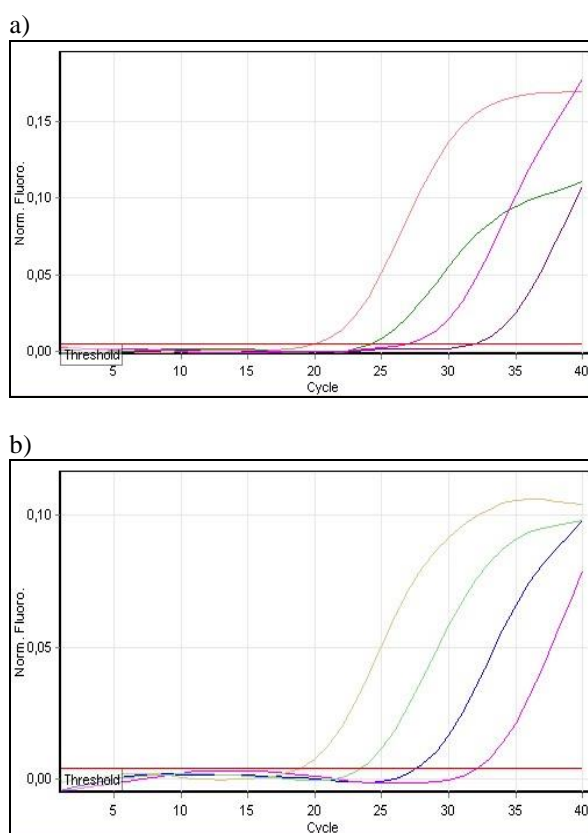
Fig. 2. Determination of the sensitivity of SYBR Green I real-time PCR with PA-F/R primers for the detection of *B. anthracis* strain 1/47 in liver (a) and blood (b) samples using Genomic Mini or Genomic Blood Mini Kits for DNA isolation

Subsequently real-time PCR detection of the *capC* gene located on plasmid pXO2 was performed. For DNA isolated from contaminated liver samples with the use of the Genomic Mini and the DNeasy Blood and Tissue Kit, Ct values ranged from 23.60 to 27.51 and from 16.72 to 18.43, respectively. Regarding DNA isolated from contaminated blood samples using the Genomic Blood Mini, Ct values were from 14.46 to 19.11 and using the DNeasy Blood and Tissue Kit from 9.56 to 14.95. Again one of the five examined

B. anthracis strains (B.a. 13/93) did not show any reaction product, which indicates absence of the capsule. Specific amplification products dissociated at about 76°C. For DNA isolated from contaminated liver samples using the Genomic Mini the sensitivity was 3.5×10^3 CFU/reaction and using the DNeasy Blood and Tissue Kit 3.5×10^2 CFU/ reaction. For DNA isolated from contaminated blood samples with the use of kits from the both manufacturers, the sensitivities were 3.5×10^2 CFU/reaction and 3.5×10^1 CFU/reaction, respectively (Fig. 3). For template DNA obtained from experimentally contaminated biological samples using the A&A Biotechnology and Qiagen kits, a high regression coefficient above 0.99 was demonstrated.

a)

b)
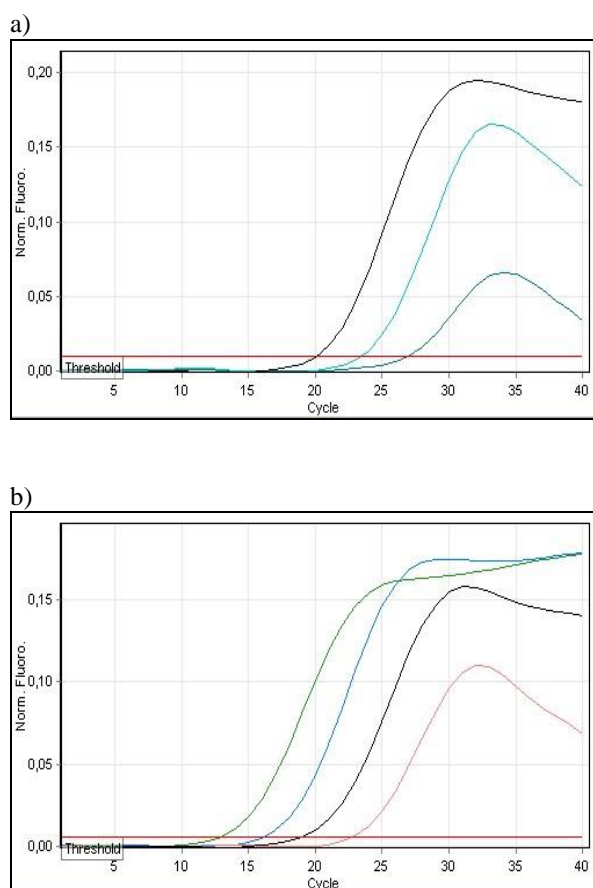
Fig. 3. Determination of the sensitivity of SYBR Green I real-time PCR with capC-F/R primers for the detection of *B. anthracis* strain 1/47 in liver (a) and blood (b) samples using a DNeasy Blood and Tissue Kit for DNA isolation

Examination of a range of bacterial DNA was undertaken to demonstrate the specificity of all SYBR Green I real-time PCRs. The template DNA was obtained from liver and blood samples experimentally contaminated with five strains of *B. anthracis*, single strains of other species of the genus *Bacillus*, *i.e.* *B. cereus*, *B. brevis*, *B. subtilis*, and *B. megaterium*, and other microbial species including *Staphylococcus aureus, Listeria monocytogenes, Pasteurella multocida,* *Escherichia coli*, *Salmonella* Typhimurium, and *Klebsiella pneumoniae*. All sets of primers were highly specific. Products of amplification specific for *B. anthracis* were obtained in all real-time PCRs, while isolates of other species failed to yield real-time PCR products.

Discussion

Identification of bacteria using traditional phenotypic methods is limited in comparison to genotypic methods. Tests based on the analysis of nucleic acids allow the direct detection of the microorganism in bacterial cultures or clinical samples. The PCR and real-time PCR techniques for identifying microorganisms at the strain, species, or genus level save time and increase sensitivity, and these methods have been used for fast and sensitive detection of *Bacillus anthracis*. Specific primers detect *B. anthracis* genes located on the DNA of plasmid pXO1, including the *pag*, *lef,* and *cya* genes (1, 16, 18, 21, 22). The second plasmid, pXO2, contains the sequences of the *cap* gene (A, B, and C) responsible for the production of the capsule (8, 19, 30). The confirmation of the presence of the two plasmids in *B. anthracis* indicates the virulence of the strain tested and has diagnostic value (7, 11, 21). Specific chromosomal markers for *B. anthracis* including Ba813 and *rpoB* are also available (7, 17, 25, 26). The conventional PCR and real-time PCR tests used in laboratories are valuable diagnostic tools. The use of real-time PCR may resolve most problems connected with the classical PCR method. This technique is less time consuming, reduces the risk of contamination of the sample due to the lack of post-amplification treatment, and may increase the sensitivity and efficiency of the method (9, 13, 20, 29). The optimisation of reaction conditions is crucial for the effectiveness of real-time PCR. The optimal concentration of the pair of primers was determined by testing different concentrations to achieve the lowest Ct value with the lowest primer concentration.

Khandia *et al.* (14) obtained a detection limit of 2×10^{-6} pg DNA, which corresponded to 3.47 copies for a pure culture SYBR Green I real-time PCR using a *pag* target. Ellerbrok *et al.* (7) achieved a detection limit of 10 plasmid copies per reaction for pure cultures in a TaqMan real-time PCR for the detection of the *pag*, *capC*, and *rpoB* genes. In our research, the results of SYBR Green I real-time PCRs for detection of the chromosomally encoded *rpoB* and the *pag* genes for DNA isolated from experimentally contaminated liver and blood samples using kits from A&A Biotechnology and Qiagen were comparable. Only in the case of real-time PCR detecting the *capC* gene slightly lower sensitivity was achieved. The reaction sensitivity for liver samples using Cap-S and Cap-R primers ranged from 3.5×10^2 to 3.5×10^3 CFU/reaction and for blood samples from 3.5×10^1 to 3.5×10^2 CFU/reaction.

Khandia *et al.* (14) obtained a regression coefficient higher than 0.989 for pure cultures in SYBR Green I real-time PCR using the *pag* gene. For the detection of the *cap* gene, Bentahir *et al.* (2) achieved a regression coefficient higher than 0.995 for pure cultures. In our studies, the high regression coefficient attained in SYBR Green I real-time PCRs with the use of the *rpoB*, *pag*, and *capC* genes indicates a high degree of reaction linearity.

In our examinations, evaluation of real-time PCRs showed the high specificity of the tests. Products specific to *B. anthracis rpoB* gene as a chromosomal marker, *pag* gene, and *capC* gene were obtained. Isolates of various species of the genus *Bacillus* and strains of six other microorganisms gave no real-time PCR products to be found. Similarly, Bode *et al.* (4) and Qi *et al.* (25) examining *B. anthracis* strains and other species of the genus *Bacillus* showed 100% specificity of the reaction. In contrast, Ellerbrok *et al.* (7) demonstrated the presence of the *rpoB* gene in four of the five *B. cereus* strains tested. Although the researchers used a comparable number of bacteria for the reaction, the Ct values obtained for *B. cereus* were found 10 cycles later than in the case of *B. anthracis*. It may be a result of the close relationship of these species (4, 7).

Summing up, the real-time PCR method based on the fluorescence of SYBR Green I light-emitting dye in intercalation with double-stranded DNA successfully detected genes located on the pXO1 and pXO2 plasmids and the *rpoB* gene as a chromosomal marker. It was shown that real-time PCRs revealed considerable sensitivity and specificity. The specificity of the tests was confirmed by the melting points of the PCR products, and the sensitivity and linearity of the reaction were determined using the regression coefficient (29).

In order to fully utilise the advantages of the real-time PCR method, TaqMan probes will be used for further identification of *B. anthracis* from experimentally contaminated biological samples. The use of fluorescently labelled probes may additionally increase the sensitivity and specificity of the method (9, 29).

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Animal Rights Statement: None required.

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