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Review article

Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores

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Abstract

To establish the rapid detection method of airborne bacterial spores, we examined *Bacillus anthracis* spores by real-time PCR. One hundred liters of air were trapped on a filter of an air monitor device. After it was suspended in PBS, spores of *B. anthracis* were artificially added. The suspension was also heated at 95 °C for 15 min and used for real-time PCR using anthrax-specific primers. A single cell of *B. anthracis* was detected by real-time PCR within 1 h. Our results provide evidence that anthrax spores from the atmosphere can be detected rapidly, suggesting that real-time PCR provides a flexible and powerful tool to prevent epidemics.

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Keywords: Real-time PCR; Airborne microorganism; Spore; Anthrax

1. Introduction

Many kinds of microorganisms exist in the air, which are mostly non-pathogenic to humans. However, though it may be artificial or rarely occur, pathogenic microbes sometimes exist in the air and cause respiratory infection. They usually exist in the air as small particles like aerosol, spore or microorganism-binding dust, but most of them will drop on the ground soon because of their own weights. However, they are floating in the air for hours if their diameters are about 1–3 μm and if they are in dried states. Therefore, the spore forming of microorganisms are most suitable for airborne pathogens. Especially, *Bacillus anthrax* is important because of their pathogenicity, meaning that anthrax spores have been assumed as the most effective candidate for the biological weapon for over 80 years and had been used actually in 2001.

Three forms of human anthrax are known: cutaneous, gastrointestinal and pulmonary (inhalation) anthrax. The cutaneous form is often self-limiting, but inhalation anthrax is sometimes severe because antibiotics only suppress the infection if administered shortly after exposure (usually within the first 24–48 h). If not treated by the time the symptoms develop, death is likely to occur in 99% of cases in unprotected individuals (James et al., 1998). Although inhalation anthrax is generally contracted from breathing in airborne anthrax spores, monitoring the exposure of *Bacillus anthracis* spores in the atmosphere is extremely difficult because the spores are not visible to the naked eye, and are colorless, odorless and taste-

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less. Therefore, a rapid and sensitive technique to detect anthrax spores in the atmosphere is important for public health and probably enough to detect various kinds of airborne microorganism by PCR. In this review, to detect rapidly anthrax spores, we show a PCR detection system using real-time PCR technology.

2. Materials and methods

2.1. Bacterial strains

Nine *B. anthracis* isolates listed in Table 1 (Makino et al., 2001) were used for the PCR. Those strains were inoculated on nutrient agar (NA; Difco Laboratory, Detroit, Mich.) plates and suspended in sterile PBS, followed by preparing their spore suspensions, as previously described (Uchida et al., 1985).

2.2. Isolation of bacterial cells from air samples

One hundred liters of air were trapped on a 0.45-μm nitrocellulose membrane filter using an aerosol analysis monitor (Millipore, Tokyo, Japan), followed by suspending in 1 ml of sterilized PBS or water. As an option, an aliquot of the sample can be inoculated in the appropriate agar plate and broth to detect the organisms, and air-samplers supplied from some companies using NA or blood agar plates are convenient. For *B. anthracis*, blood agar supplemented with polymyxin B, PLET plates (Knisely, 1966) or *Bacillus cereus* selection agar (BCA; Oxoid, Hampshire, England or Merck, Japan) are available.

Table 1
Bacillus strains used in this study

Bacillus strains	PA	CAP
Shikan	+	+
Morioka	–	+
Pasteur II	+	+
Pasteur I	+	+
Nakagawa	–	+
Ryugasaki	+	+
52	+	+
P-I	+	+
34-F2	+	–

2.3. Preparation of DNA samples for PCR

The suspension is centrifuged at 15,000 rpm for 5 min and resuspended in 10 μl of sterile water, followed by heating at 95–100 °C for 15 min and centrifugation at 15,000 rpm for few minutes at 4 °C, and then 1 μl of the supernatant was directly used for PCR. Since spores might be contained in the samples, a class IIb safety cabinet should be used for the preparation.

2.4. PCR

Oligonucleotide primers for PCR were as follows: specific primers (CAP primers) to amplify a 591-bp DNA fragment of the *cap* region essential for encapsulation (Makino et al., 1989), MO11 (5′-GACGGATTATGGTGCTAAG-3′) and MO12 (5′-GCACTGGCAACTGGTTTTG-3′); specific primers (PA primers) to amplify a 211-bp fragment of the *pag* gene to produce a component of the toxin (Price et al., 1999), PA7 (5′-ATCACCAGAGGCAAGACACCC-3′) and PA6 (5′-ACCAATATCAAAGAACGACGC-3′). For standard PCR, all primers, 20 pmol each, were mixed in a reaction tube, and PCR was performed in a reaction mixture (25 μl) using model 9600 (Applied Biosystems Japan, Tokyo, Japan). The following PCR cycles were used: 1 × 95 °C for 2 min; 35 × (95 °C for 15 s followed by 60 °C for 15 s followed by 72 °C for 30 s); 1 × 72 °C for 5 min; cool to 4 °C. The real-time PCR was performed using two systems of Light Cycler (Roche Diagnostic, Tokyo Japan) and Smart Cycler (Takara shuzo, Kyoto Japan), which was originally from Cepheid, CA, USA. PCR amplification was performed according to the supplier's instructions. For the Light Cycler system, Light Cycler™ FastStart DNA Master SYBR Green I (Roche Diagnostic) was used. For the Smart Cycler system, TaKaRa *B. anthracis* PCR Detection Kit (Real-Time PCR Version) (Takara shuzo) was used, which consisted of each primer set as described above and internal control to prevent false positive; in the negative reaction, 152 and 395 bp PCR products with 80 and 82 °C melting peaks using CAP and PA primers, respectively. The following PCR cycles were used: 1 × 95 °C for 10 min; 40 × (95 °C for 10 s followed by 64 °C for 10 s followed by 72 °C for 20 s) for the Light Cycler and 1 × 95 °C for 30 s;

40 × (95 °C for 5 s followed by 68 °C for 30 s) for the Smart Cycler.

3. Results and discussion

3.1. Standard PCR Technology

Generally, 10–1000 bacterial cells in the suspension prepared from 100 l of air should be detected on the NA plates. When anthrax spores were artificially contaminated into such suspensions followed by spreading on the BCA plates, background bacterial cells grew, but large rough colonies of *B. anthracis* cells were detected on the plates (Fig.1). These rough colonies were subsequently confirmed by PCR.

Standard PCR with two sets of primers was performed using the DNA samples prepared, showing that those primers amplified the right sizes of two DNA fragments using DNA samples containing over 10 spores, but that the DNA fragments detected were faint using DNA samples containing only one spore (Fig. 2, panel A, lane 2).

3.2. Light Cycler

The conditions for real-time PCR using Light Cycler were found using DNA samples prepared as in the standard PCR and CAP primers. The highest annealing temperature was the temperature at which no amplified fragment was detected using a sample with no spores. Thus, the PCR cycle for the Light Cycler system was determined as described in Materials and methods (Fig. 3, panel A). Simultaneously, the fluorescence signals were measured using the quantification program of Light Cycler software version 4.2 (Roche Diagnostics). As the copy number of the *cap* gene correlates with numbers of spores, the relative numbers of spores in the samples should be estimated by measuring the fluorescence signals. When the copy numbers of the *cap* gene in the samples containing one spore was one, the relative copy number of the *cap* gene in the samples containing 10 and 100 spores were estimated as 9.95 and 100.02, respectively. Thus, when samples containing known numbers of cells were used as standards in real-time PCR, the number of spores in a sample can be estimated.

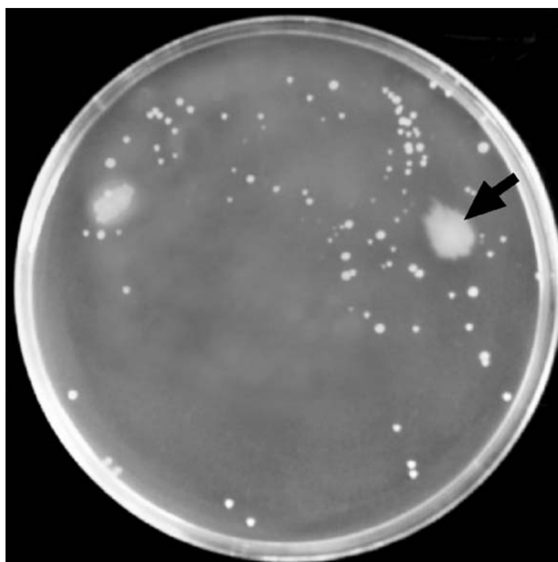

Fig. 1. Isolation of anthrax spores on a BCA plate. The suspension from 100-l air sample containing one spore was plated on a BCA plate. The arrow shows a colony of *B. anthracis*.

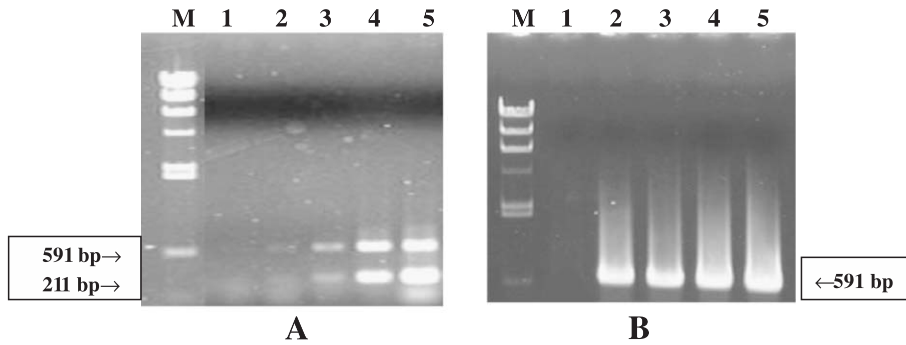

Fig. 2. Detection of anthrax DNA from air samples using PCR. Template DNAs were prepared from air samples containing 0 (lane 1), 1 (lane 2), 10 (lane 3) and 100 (lane 4) anthrax spores, and then were used for standard PCR (A) and real-time PCR (B). M, λ DNA digested with HindIII as the molecular size marker; lane 5, purified DNA.

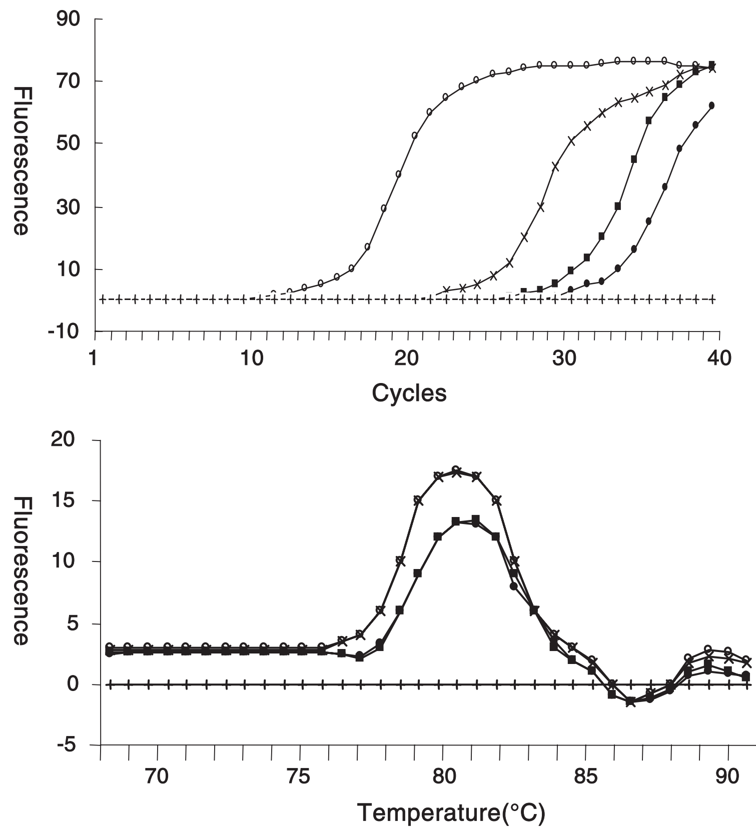

Fig. 3. Fluorescence graph (A) and melting curve (B) using the Light Cycler system. O—purified DNA; ×—100 spores per sample; ■—10 spores per sample; ●—1 spore per sample; +—0 spore per sample.

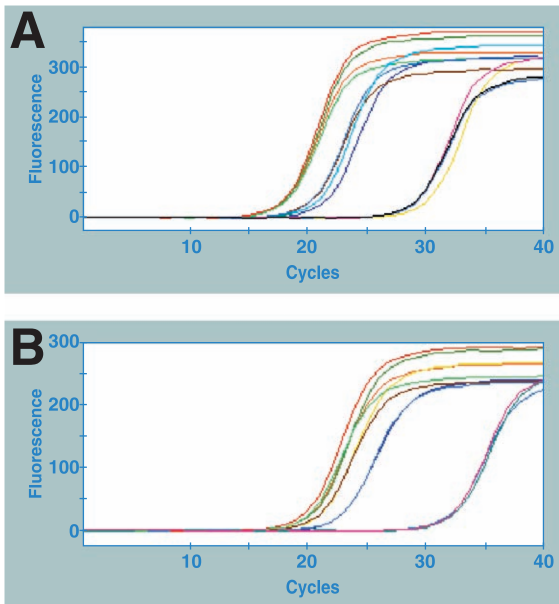

Fig. 4. Fluorescence graphs using PA (A) and CAP (B) primers in the Smart Cycler system. Internal control was always amplified after 30 cycles in negative samples.

To analyze the melting curve, the PCR products was set up with a target temperature of 95 °C using Light Cycler software version 4.2, which was performed immediately after the amplification finished. This program can differentiate between signals obtained from specific PCR products and nonspecific DNA fragments, such as primer-dimer, and thus, specific PCR products must show the same melting peaks. In this study, the melting peaks of the amplified products were about 84.5 °C (Fig. 3, panel B), showing that we could recognize amplified specific products without electrophoresis. To confirm that PCR was successful, the amplified products were electrophoresed in agarose gel. Specific PCR products were detected, even in the sample containing one spore (Fig. 2, panel B). The same PCR conditions and results were obtained using PA primers except with a melting peak of about 82 °C (data not shown).

3.3. Smart Cycler

The real-time PCR with Smart Cycler was performed using airborne samples, which were artificially contaminated with each 100 spores from nine strains, by a new PCR kit, and then the fluorescence signals were measured using those samples. The signals were detected at about 20 cycles in positive samples, but the signals, which were generated from the internal control, were detected at about 30 cycles in negative samples (Fig. 4).

To analyze the melting curve, the PCR products was set up with a target temperature of 95 °C. In this study, the melting peaks of the amplified products using PA primers were about 82 °C in positive samples and about 80 °C in negative samples (Fig. 5, panel A). The peaks using MO primers were about 85 °C in positive samples and about 82 °C in negative samples (Fig. 5, panel B), showing that we could recognize amplified specific products without electrophoresis. To confirm that PCR was successful, the amplified products were electrophoresed in agarose gel. Specific PCR products of 211 and 152 bp in positive and negative samples, respectively, were detected using PA primers (Fig. 6. panel A), and also positive 591 bp and negative 395 bp DNA fragments were detected using CAP primers (Fig. 6, panel B).

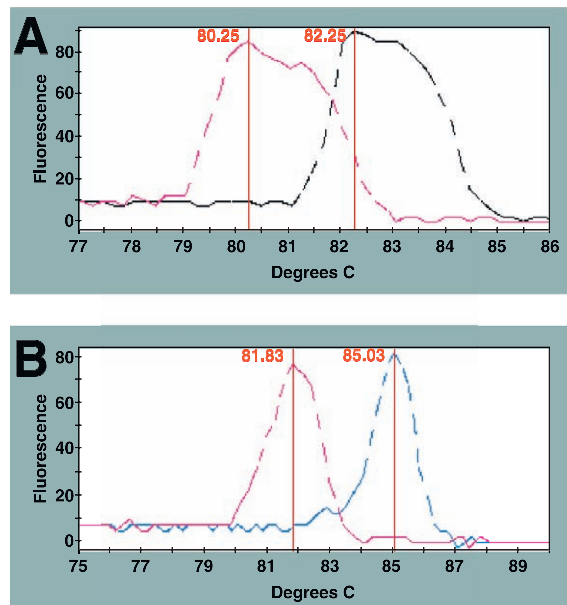

Fig. 5. Melting curves using PA (A) and CAP (B) primers in the Smart Cycler system. Melting peaks of the PCR products amplified by PA and CAP primers were about 82 and 85 °C, respectively. Lower peaks were derived from the PCR products amplified by the internal control.

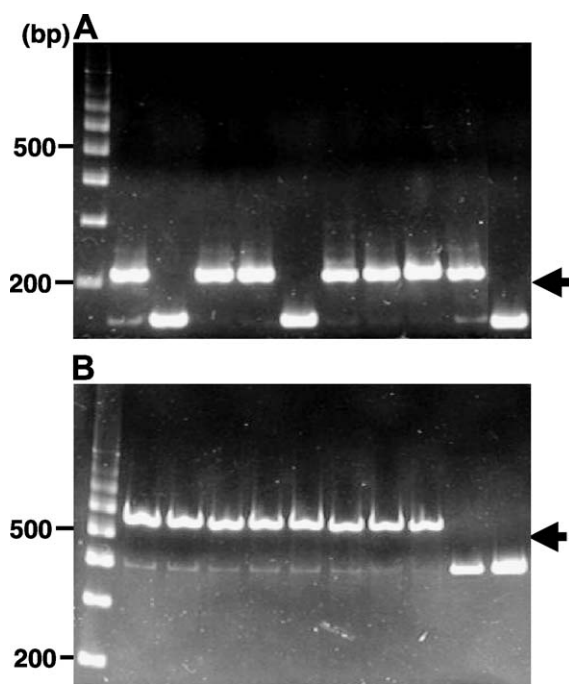

Fig. 6. Detection of anthrax DNA from air samples using the Smart Cycler system. Template DNAs were prepared from air samples containing about 100 anthrax spores of Shikan (lane 1), Morioka (lane 2), Pasteur II (lane 3), Pasteur I (lane 4), Nakagawa (lane 5), Ryugasaki (lane 6), 52 (lane 7), P-I (lane 8) and 34-F2 (lane 9) using PA (A) and CAP (B) primers. M, 100 bp DNA ladder; lane 10, without spores. The arrow shows PCR product in the positive sample. Shorter products were amplified by the internal control in the negative sample.

We showed that template DNA for PCR could be prepared from air samples within 1 h by heating. We also purified template DNA using a DNA extraction kit (Qiagen K.K. Japan, Tokyo) with the results of PCR identical to those by heating, but preparing template DNA using this kit took about 2 h. From the results, heat treatment alone proved sufficient to prepare template DNA for PCR and had the advantage of a shorter time than using the kit.

Target fragments were detected within 4 h using the standard PCR system, and within 1 h using the real-time PCR system, which also had the advantage of making it possible to estimate the number of anthrax spores in the air and to confirm the amplification of target products using only melting curve analysis without agarose gel electrophoresis. In addition, the PCR kit used in this study might be more convenient because of an internal control. Generally, detecting *B. anthracis* infection and diagnosing anthrax in humans is difficult because early symptoms are nonspecific. However, monitoring anthrax spores in the environment, especially in the air, can aid detection and prevent *B. anthracis* infections in humans. This real-time PCR system using the Light Cycler is a flexible and powerful tool to prevent epidemics because it can detect one spore in an air sample. Also, this real-time PCR system can detect *B. anthracis* cells from food, drink and soil, which are thought to be other sources of anthrax. However, preparation of template DNA for PCR would be more difficult from these other sources than from air samples because of the large number of cells of various bacterial species, organic compounds, etc. (manuscript in preparation).

In this study, we showed that one *B. anthracis* spore in 100 l of air sample can be detected using PCR and we also showed the isolation of one spore on a

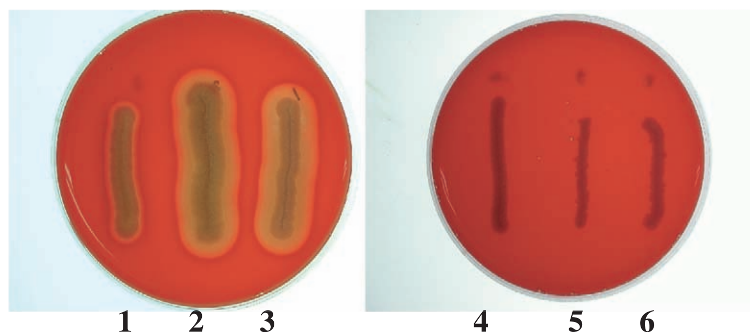

Fig. 7. Haemolysis on blood agar plates of *Bacillus* strains. *B. cecreus* (1), *B. mycoides* (2), *B. thuringiensis* (3) and *B. anthracis* (4–6) strains were streaked on blood agar plates supplemented with the BCA supplement. Those plates were incubated at 37 °C for 16 h.

BCA plate. As we can isolate *B. anthracis* vegetative cells from meat and tissue effectively using BCA plates (Cheun et al., 2001), a BCA plate would be a effective tool to detect *B. anthracis* from various samples. At the same time, blood agar plates supplemented with the same supplement used with BCA were also effective because hemolysis was easily detected (Fig. 7). Finally, for the initial examination, real-time PCR and direct plating onto selection plates would have to be done at the same time. If the samples contain more than one spore, the PCR method can detect the existence of *B. anthracis* spores in the air within 1 h and can isolate *B. anthracis* cells within 2 days. Such an automatic monitoring system for *B. anthracis* might be an essential tool for epidemiological surveys and for the prevention of *B. anthracis* infections in the future. In this article, we showed the application of the real-time PCR only in anthrax spores, but this procedure should be available to detect rapidly other airborne microorganisms.

Acknowledgements

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