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Short Communication

Preparation of a Positive Control DNA for Molecular Diagnosis of *Bacillus anthracis*

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SUMMARY: *Bacillus anthracis* is considered to be one of the most potent biological weapons because of its highly pathogenic nature and efficiency of transmission. Routinely, a presumptive diagnosis of anthrax is achieved if the bands with predicted sizes are detected after the PCR targeted to the *pag* and *cap* genes residing on pXO1 and pXO2 plasmids, respectively. A positive control DNA prepared from the standard strains of *B*. *anthracis* (PAI and PAII) is usually included in the PCR tests. The handling of living *B*. *anthracis*, however, requires physical containment. The inclusion of DNA from *B*. *anthracis* as a positive control in the PCR test also has a potential risk of cross contamination that may confuse the results. In order to circumvent such problems, we attempted to construct a recombinant plasmid harboring the fragments of the *pag* and *cap* genes that could be distinguished from authentic sequences by the presence the restriction-enzyme site, the *Eco*RV site for the *pag* gene and the *Bam*HI site for the *cap* gene, respectively, which were newly introduced. The strategy reported here provides a safe and reproducible positive-control DNA template. It also allows the detection of possible cross contamination, indicating that this strategy would be useful and convenient for the molecular identification of not only *B*. *anthracis* but also other highly pathogenic microbes.

Bacillus anthracis is considered to be one of the most potent biological weapons because of its highly pathogenic nature and efficiency of transmission (1). After the anthrax attack by bioterrorists in the United States (U.S.) in September 2001, thousands of envelopes containing so-called "white powder" were disseminated to many places not only in the U.S. but also in Japan. Public health institutions are responsible for testing these envelopes for the presence of *B*. *anthracis* even though the majority of them are hoaxes, demonstrating that rapid and precise diagnostic methods to detect anthrax are urgently needed.

In Japan, the PCR method recommended by the World Health Organization (WHO) is accepted as the standard diagnostic approach for identifying *B*. *anthracis* (2). Routinely, a presumptive diagnosis of anthrax is achieved if the bands with predicted sizes are detected after the PCR is targeted to *pag* and *cap* genes residing on pXO1 and pXO2 plasmids, respectively (3). A positive control DNA prepared from the standard strains of *B*. *anthracis* (PAI and PAII) is usually included in the PCR tests. The handling of living *B*. *anthracis*, however, requires physical containment. The inclusion of DNA from *B*. *anthracis* as a positive control in the PCR test also has a potential risk of cross contamination that may confuse the results. In order to circumvent such problems, we attempted to construct a recombinant plasmid DNA harboring the fragments of the *pag* and *cap* genes that could be distinguished from authentic sequences by the presence of the restriction-enzyme site, the *Eco*RV site for the *pag* gene, and the *Bam*HI site for the *cap* gene, respectively, which were newly introduced.

For introduction of each restriction-enzyme site into the DNA fragments of the *pag* or *cap* genes, overlap extension PCR (Table 1 and Fig. 1) was applied (4). DNA extracted from *B*. *anthracis* (PAI strain) was used as a template DNA for the PCR reaction. In short, the first PCR was performed using the forward primer A and the reverse primer B in order to obtain the left half of the *pag* gene fragment. The primer B was designed to contain a restriction site recognized by *Eco*RV. The right half of the target was amplified with the primer C containing the same restriction-enzyme site and the primer D. The resulting amplicons were used as a template to generate the DNA fragment containing the *Eco*RV site in the

Table 1. Primers used in this study

Primers	Sequence (5′-3′)
pag gene (based on accession no. AF065404)	
PA5	tcctaacactaacgaagtcg
PA8	gaggtagaaggatatacggt
primer A	caagtatttgacttaattgg
primer B	gtacatgtagatatCgagaatattattc
primer C	ttctcGatatctacatgtacaatcg
primer D	aagcgaagtacaagtgctgg
cap gene (based on accession no. AF188935)	
CAP1301	tcccacttacgtaatctgag
CAP1234	ctgagccattaatcgatatg
primer A'	aggatgttcgaaattcccac
primer B'	ggtgtttcaGgatCcatgattttatatg
primer C'	atcatgGatcCtgaaacaccatacg
primer D'	attatgtctcgtatgcgtcc

Primer pairs of (PA5 and PA8) and (CAP1301 and CAP1234) were diagnostic primers recommended by the WHO. Primer sets of A, B, C and D & A', B', C' and D' were used for the overlap extension PCR of the *pag* and *cap* genes, respectively. The generated restriction enzyme sites are indicated by underline. Primers B and C have a *Eco*RV site (gatatc) and primers B' and C' have a *Bam*HI site (ggatcc).

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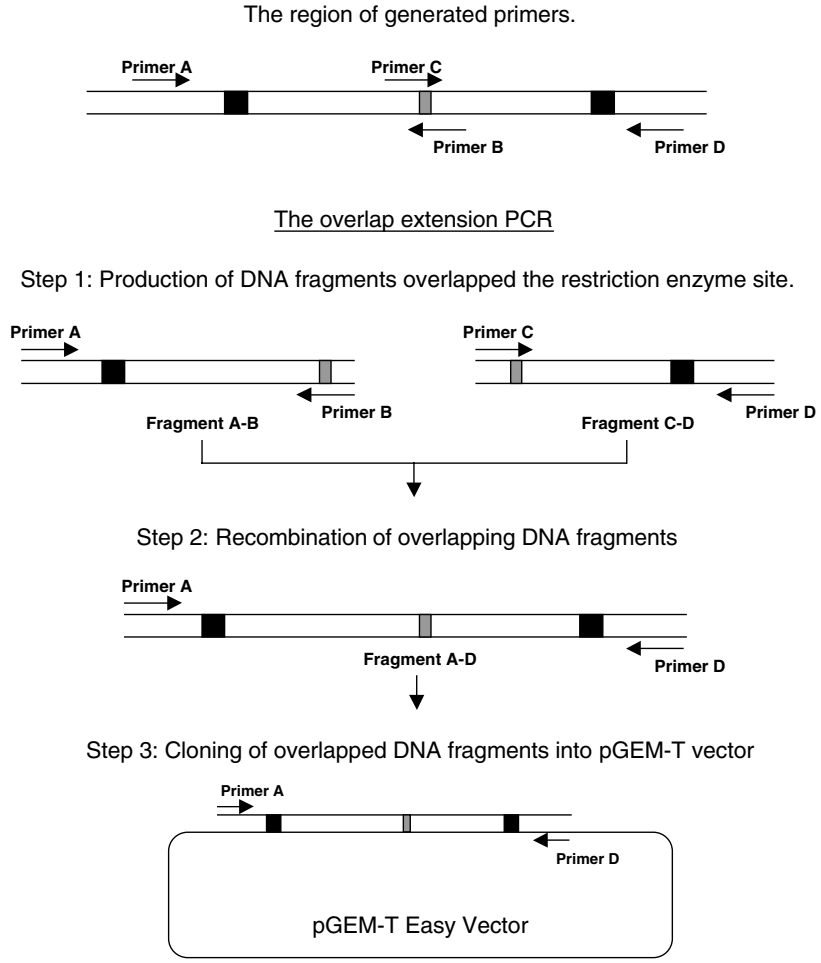

Fig. 1. Construction of recombinant plasmid DNAs harboring the mutated DNA fragments of the *pag* gene that were flanked by primer pairs recommended by the WHO. Step 1 was carried out using the primers. PCR reaction of Step 2 was started using a mixture of fragments of A-B and C-D (each 20 ng) purified by means of QIAquick Gel Extraction Kit (QIAGEN). The first 5 cycles of PCR were performed without using primers of A and D, and then next 35 cycles were performed using these primers.

middle of the sequence by PCR using the primers A and D. Introduction of the *Bam*HI site into the *cap* gene fragment was similarly conducted. These PCR fragments were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and ligated into a cloning vector, pGEM-T (Promega, Madison, Wis., USA), by means of a TA-ligation method. The resulting recombinant plasmid DNAs designated pGEM-PAc1 for the *pag* gene and pGEM-CAPc3 for the *cap* gene, respectively, were subjected to nucleotide sequencing to ensure that each restriction site was properly introduced into them (Fig. 2). These replacements of the nucleotide did not affect the results of the PCR assays for the detection of both the *pag* and *cap* genes. A nucleotide at position 130 was changed from T to C in the insert of the *pag* gene, and three nucleotides at positions 8, 52, and 591 were changed from T, A, and A to C, G, and a deletion in the insert of *cap* gene, respectively. The nucleotide sequences were determined using a Thermo Sequenase Cy5 Dye Terminator kit according to the manufacturer's instructions and with a Long-Read Tower DNA sequencer (Amersham Pharmacia Biotech Inc., Piscataway, N.J., USA). The sequence data of the inserted DNA fragment of pGEM-PAc1 and pGEM-CAPc3 will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers AB125961 and AB125962, respectively. Although it was found that a nucleotide at position 591 was deleted from the target region of pGEM-CAPc3. This deletion did not affect the recognition by the enzyme.

The pGEM-PAc1 and pGEM-CAPc3 plasmids were subjected to PCR amplification by the diagnostic primer pairs (PA5 and PA8 for the *pag* gene and CAP1301 and CAP1234 for the *cap* gene) (Table 1) recommended by the WHO, respectively. PCR reaction was performed using *Ex Taq* polymerase (TAKARA BIO Inc., Shiga) according their protocol employing an automated thermal cycler (ASTEC PC808, ASTEC Inc., Fukuoka) for 35 cycles (each consisting of 15 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C) after denaturation for 5 min at 95°C. PCR mixtures contained 2.5 U of *Ex Taq* polymerase, 10 pmol of each primer, and 10 ng of template DNA in a 50 μl total reaction volume. The PCR products obtained by the amplification of pGEM-PAc1 and pGEM-CAPc3 as the template, respectively, were incubated with either *Eco*RV or *Bam*HI. These digests yielded two distinct bands 366 bp and 237 bp for the *pag* gene, and 570 bp and 282 bp for the *cap* gene, while PCR products amplified from the DNA extracted from PAI strain of *B. anthracis* showed only the one band even after these products were digested by *Eco*RV or *Bam*HI (Fig. 3).

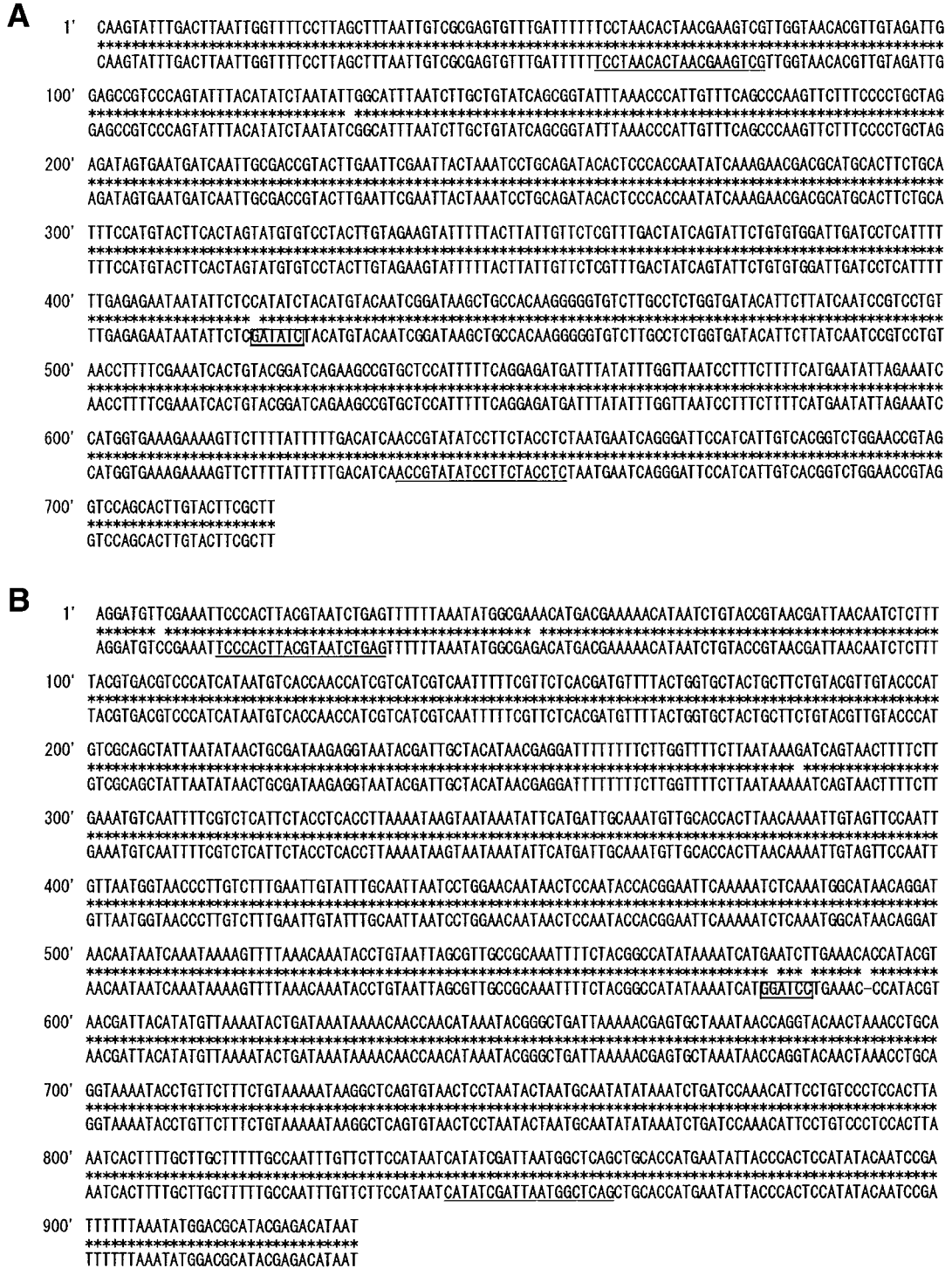

Fig. 2. The nucleotide sequences of the inserted region of pGEM-PAc1 (A) and pGEM-CAPc3 (B). The sequences of A-D and A'-D' fragments obtained in this study (lower) were aligned with those of the *pag* and *cap* genes (upper) assigned in GenBank as accession numbers AF065404 and AF188935, respectively. The generated restriction sites are encircled by solid lines. The sequences of primers reported by the WHO are underlined (PA5, PA8, CAP1301, and CAP1234).

Inclusion of the positive control when diagnosing anthrax by means of PCR is a sort of prerequisite to ensure that the primers and other aspects of the process are working. The preparation of the DNA from the bacteria, however, requires a Biosafety Level 3 laboratory, and handling of highly pathogenic *B. anthracis* may impose a potential risk of infection. In addition, cross contamination of the control DNA could compromise the results.

The strategy described here provides a safe and reproducible positive-control DNA template. It also allows the detection of possible cross contamination, indicating that the strategy would be useful and convenient for the molecular diagnosis of not only *B. anthracis* but also other highly pathogenic microbes.

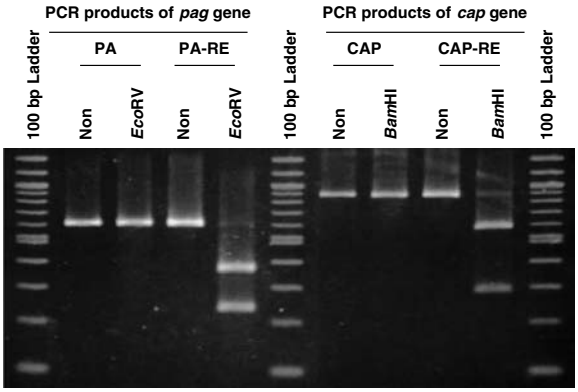

Fig. 3. The target sequences of *pag* and *cap* genes were amplified by means of the primer pairs of PA5 & PA8 and CAP1301 & CAP1234, respectively. PA and CAP indicate the PCR products amplified from the DNA-extracts of the *B. anthracis* PAI strain, whereas PA-RE and CAP-RE indicate the PCR products amplified from pGEM-PAc1 and pGEM-CAPc3, respectively. The PCR products of the *pag* and *cap* genes were digested by the restriction enzymes, *Eco*RV and *Bam*HI, respectively. The molecular marker was a 100 bp Ladder. The sample was loaded on 4% agarose gel.

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