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Detection of BCG Vaccine by Capillary Electrophoresis

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ABSTRACT

We have used the polymerase chain reaction (PCR) to amplify specific DNA fragments of the *Mycobacterium bovis* BCG (BCG) vaccine from genomic DNA. We have used capillary electrophoresis (CE) to analyze and detect the PCR products. The ability to amplify a DNA fragment of about 350 bp will bring the speed and simplicity of PCR to vaccine identification in a genomic DNA, and even to quantitative DNA research. While current methods for determining the identity of BCG vaccine are very labor intensive, in this paper we will describe a PCR and CE technique for identifying the BCG vaccine. Thus, the identification of PCR products by CE may offer better resolving power, convenience, and quantitative capability than the conventional electrophoresis methods.

Key words: BCG Vaccine; PCR; capillary electrophoresis.

INTRODUCTION

BCG is an attenuated derivative of *Mycobacterium bovis*, a virulent tubercle bacillus very closely related to *M. tuberculosis*. The current control methods recommended by the EP[(1)] and WHO[(2)] to confirm the identify of BCG vaccines involve a microscopic examination of the bacilli in stained smears, demonstration of their acid-fast properties, and determination of the characteristic appearance of colonies grown on solid medium. The routine culture of BCG vaccine is cumbersome and time-consuming. While microscopic examination of smears of acid-fast bacteria by Ziehl-Neelsen staining is the quickest method of detecting mycobacteria, it is insensitive and nonspecific. A major limitation to immunological detection of infections with *M. tuberculosis* by nonculture methods, such as latex agglutination, radioimmunoasssay, and enzyme-linked immunosorbent assay, is their lack of sensitivity and/or specificity [(3,4,5)]. Although serological techniques may be useful in some clinical settings, this approach is limited, in general, due to poor sensitivity or specificity[(6,7,8)]. For this reason, the development of another rapid method to identify the vaccine would be highly desirable. Recent advances in DNA techniques have provided a new approach to the rapid diagnosis of mycobacterial disease through the use of species-specific nucleic acid probes[(9,10,11)]. The recently developed polymerase chain reaction, which permits the in vitro amplification of DNA segments, has been shown to increase the level of detection enormously[(1,2,5,7,12-19)]. The purpose of the present work is to describe the isolation of BCG vaccine DNA by PCR amplification, while also attempting to apply CE techniques to DNA isolation.

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Capillary electrophoresis offers the advantages of fast run times and ease of sample introduction[(3)]. The main disadvantage of capillary gel electrophoresis, however, is that it is a serial technique, and as a result, its total outcome is no better than that of conventional gels, despite their long run times and need for parallel separation. In this paper, we will investigate the use of electrophoresis with capillary sample introduction as a simple, rapid, and reliable method of PCR analysis.

MATERIALS AND METHODS

I. *Bacterial strains*

The bacterial strains used in this study include BCG Tice, BCG Taiwan, BCG Brazilian, BCG Connaught, BCG Danish, BCG Glaxo, BCG Japanese, BCG Pasteur, BCG Russian.

II. *Media and Reagents*

BCG cultures were grown in Middlebrook 7H9 medium (Difco)supplementd with albumin-dextrose complex (ADC; Difco) and 0.05% Tween 80 (M-ADC-TW broth) at 37˚C in an incubator.

III. *Preparation of DNA templates*

(I) *Boiled cell lysates*

After incubation, one mL of a bacterial culture was pipeted into a sterile 1.5 mL polypropylene tube and centrifuged 8000 Xg for 10 min. The pelleted material was washed twice with phosphate buffered saline (PBS), resus-

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pended in 100 μL sterile water and boiled for 10 min. The cell lysates were centrifuged 8000 g for 1 min to pellet cellular debris. The supernatant was then used for the PCR. Bacterial isolates obtained from agar plates were picked and transferred to 1 mL PBS and prepared as above.

(II) *Genomic DNA isolation*

Bacterial cultures and growth of BCG isolates were harvested by mean of centrifugation and then suspended in Tris-EDTA buffer (TE; pH 8.0), heat-inactivated for 10 min at 80˚C and centrifuged at 15000 g for 15 min. The pellet was then resuspended in lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 0.5% Tween-20). Proteinase K and sodium dodecyl sulfate were added to final concentrations of 0.1 mg/mL and 1%, respectively, and incubation was continued for 1 hr at 37˚C. The mixture was extracted with a phenol-chloroform-isoamylalcohol (25;24;1,v/v/v) solution, precipitated with isopropanol (0.6 volume) and washed with 70% ethanol (2~3 times). The DNA was eluted in 200 μL of sterile distilled water. The concentration of DNA was determined by measuring the absorbance at 260 nm.

IV. *PCR and Colony PCR*

The oligonucleotide sequences of the two BCG primer pairs are BCGF1: 5'- GAA GTCGTAACAAGGTAG CCG -3'; 5'-BCGR1: GCGTAGGCGTCGGTGACAAA-3'. Each PCR mixture contained 10 mM Tris (pH 8.3), 50 mM kCl, 0.01% gelatin, 1.2 mM $MgCl_2$, 100 nM each primer, 200 μM each of the four deoxynucleoside triphosphates (dNTP) with 1U of Taq DNA polymerase (AmpliTaq DNA polymerase; Perkin-Elmer Cetus, Norwalk, Conn.), and approximately 100 ng of genomic BCG DNA as template in a thermal cycler (Perkin Elmer-Cetus, 9600). The amplifications were performed in a 50 μL of sample solution. Furthermore, 30 cycles were run for 1 min at 95˚C, 30 sec at 55˚C, and1min and 30 sec at 72˚C, with a final elongation step of 10 min at 72˚C.

Colonies were picked and transferred to 50 μL water, boiled for 5 min and stored at 4˚C until it was used. PCR amplifications were carried out by mixing 2 μL as lysate into each reaction tube. The DNA were amplified in 0.2 mL tubes for 30 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 2 min followed by a 7 min final extension at 72˚C using a Gene Amp PCR system 2400 thermal cycler (PE Biosystem, Foster City, CA. (USA). The reactions were separated by electrophoresis on a 1% TAE agarose.

V. *CE analysis*

A Beckman P/ACE 5500 CE instrument was used with the UV detection module (Beckman Instruments, Fullerton, CA). P/ACE Station software version 1.0 was used for instrument control and data collection. DNA fragments were sized by comparison to a 100 ladder of a *phix*-Hae III DNA restriction digest (Sigma, St. Louis, Mo) were not desalted, but diluted with water prior to injection to a concentration of 5 to 10 μg/mL. The DNA samples were separated at 185~740 V/cm in a 27cm × 59 μm ID coated capillary.

RESULTS AND DISCUSSION

I. *Specific DNA amplification of BCG Vaccine*

Two oligonucleotides were synthesized based on the sequence of 16S-23S rRNA. These oligonucleotides were used as primers for PCR amplification of chromosomal DNA. As expected, after amplification of either the Taiwan or Tice strain of *M. bovis* BCG DNA, a fragment of approximately 350 bp was visible on the agarose gel (Figure 1). Chromosomal DNAs of 9 BCG substrains were also found to contain the 350 bp amplificable fragment. In further PCR tests by colony, all isolates were tested by PCR, and all were shown to contain the 350 bp amplifiable fragment.

The DNA templates, prepared either by boiling or by the genomic DNA isolation method, were suitable for PCR. No significant improvement in the amplification process was observed using either method. DNA templates were prepared from isolate cultures of various substrains of *M. bovis* BCG for PCR tests. Electropherograms of both preparations were identical, and each revealed the same DNA fingerprint pattern (Figure 1). Optimization of the PCR process included varying the annealing temperatures through the range of 40˚C~60˚C. Temperatures greater than 55˚C (optimal) resulted in fewer amplification products, leading to decreased differentiation capability.

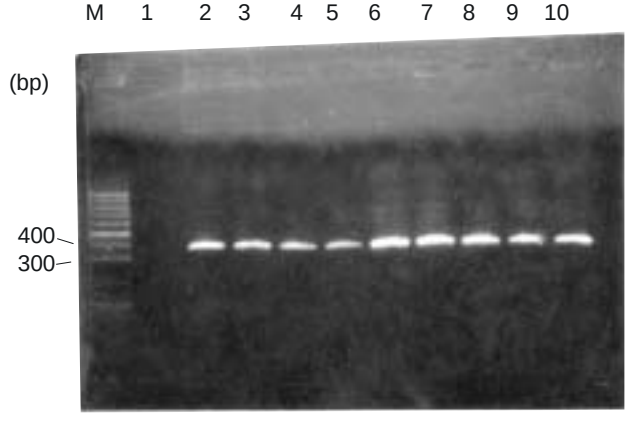

Figure 1. Amplification of DNA fragrments from different Mycobacterial BCG strains. DNA was amplified by using oligonucleotide primers BCGF1 and BCGR1 in PCR. Lane 1, Negative control with water; Lane 2, BCG Brazillian strain; Lane 3, BCG Conaught strain; Lane 4, BCG Danish Strain; Lane 5, BCG Glaxo strain; Lane 6, BCG Japanese strain; Lane 7, BCG Pasteur strain; Lane 8, BCG Russian strain; Lane 9 BCG Taiwan strain; Lane 10, BCG Tice strain. M, 100 bp DNA ladder marker. Numbers on the left indicate sizes of standard DNA fragments in base pairs.

II. *CE analysis*

In our study, we show that capillaries were effective at resolving the standard DNA *phix* DNA mixture when there was an applied voltage, i.e. 185V/cm. Figure 2A. shows electrophergrams of phix174 DNA fragment standards at 118, 194, 234, 271/281, 310, 603, 603, 872, 1078, 1358 bp. Ten peaks were obtained at 185 v/cm field strength. Tests under the same conditions, using the same applied voltage, resolved the majority of DNA products, and migration time was within 25 min in all cases. Tracking dye usually migrated between 15 and 18 min and served as an internal control for each capillary electrophoretic run. BCG from all different isolates exhibited unique DNA patterns. In the separation of two PCR products, 350 bp was the same (Figure 2B). Duplicate injection of the PCR samples yielded reproducible results. Mixtures of standard DNA and PCR products can be seen in Figure 2C.

The preparation of suitable DNA templates for PCR can be accomplished simply by boiling bacterial cells. Because of its simplicity, PCR performed directly on bacterial colonies on agar plates has been used extensively as a first means of identifying recombinant clones. CE markedly enhances the differentiation capabilities of PCR, and can clearly resolve large and small DNA fragments. As previously described[3], a low-viscosity entangled polymer systems can separate even large DNA fragments. Although sizing DNA fragments in each DNA fingerprint is possible, this would be too cumbersome. In fact, pattern comparison of each DNA fingerprint was sufficient to distinguish each BCG vaccine isolate. However, additional work must be performed to determine the amount of DNA needed for PCR to generate the same DNA patterns.

This technique is clearly advantageous for vaccine analysis of bacterial pathogens. Vaccine from bacterial isolates can be easily characterized, providing relevant information for epidemiology or microbiology. The reproducibility of PCR results was firmly established in our previous study[14], and it is plausible that electropherograms of each BCG type can serve as specific DNA templates. These template may then be used to compare all analyzed strains, further increasing differentiation capabilities. It was possible to identify each BCG strains by this technique. To speed up the analysis time, an effort should be made to find out if capillaries with shorter effective length or an increased applied voltage can be used to resolve PCR products and the DNA fragment standard solution. The PCR method coupled with CE provides a good analytical tool for characterizing bacterial vaccines. The application of this technique to other bacterial species has been successful in generating DNA patterns.

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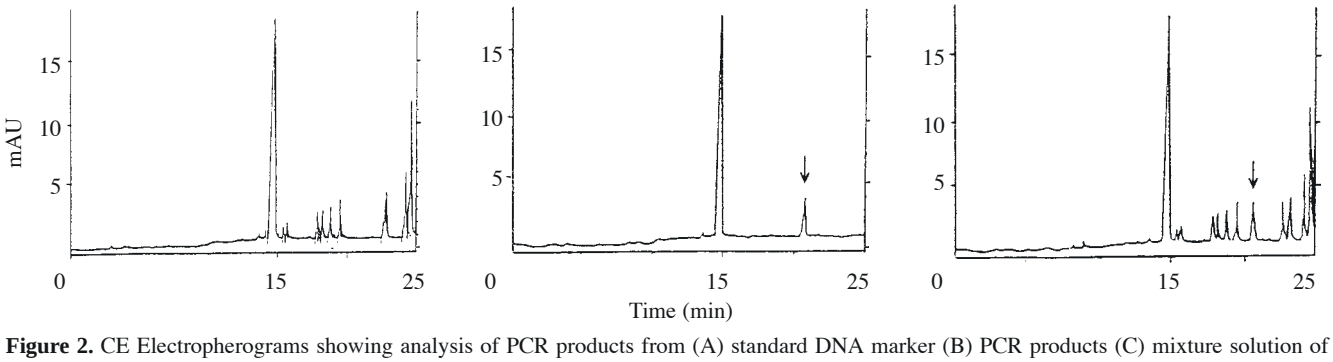

Figure 2. CE Electropherograms showing analysis of PCR products from (A) standard DNA marker (B) PCR products (C) mixture solution of standard DNA marker and PCR products. The experimental condition are provided in the text.

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