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Rapid Differentiation of BCG Vaccine from *M. smegmatis* by Genomic Amplification

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ABSTRACT

Amplification of the region of 16S-23S rRNA spacer was performed on 9 isolates of *Mycobacterium bovis* BCG (including Brazilian, Connaught, Danish, Glaxo, Japanese, Pasteur, Russian, Taiwan and Tice) and one isolate of *Mycobacterium smegmatis* using BCGF1 and BCGR1 as the primer pair. In this study we found 350-bp of PCR product in all the BCG strains but a 456-bp amplified fragment from *M. smegmatis*. Consequently, this primer pair may be a potential tool in differentiating between these two Mycobacterium species.

***Key words:* *Mycobacterium bovis* BCG, *M. smegmatis*, vaccine, polymerase chain reaction.**

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis, accounts for more deaths in the world than any other pathogen[1,2]. It is estimated that one-third of the world's population is infected with *M. tuberculosis*, and that each year 3 million people die of this disease[3]. In an effort to control the threat of tuberculosis, attenuated bacillus Calmette-Guerin (BCG) has been used as a live attenuated vaccine. BCG is an attenuated derivative of *Mycobacterium bovis*, a virulent tubercle bacillus very closely related to *M. tuberculosis*[4]. BCG has been used for over 5 decades to immunize over 3 billion people in immunization programs against tuberculosis. While its protective efficacy against tuberculosis has been highly variable, based on recent meta-anaylsis estimates, on the average, the BCG vaccine can reduce the overall risk of tuberculosis by 50% and that of serious forms of this disease by 70 to 80% [5]. As a safe, inexpensive vaccine with potent nonspecific immunostimulatory properties, BCG has more recently been proposed as a live recombinant vehicle developed for new multivalent vaccines against other diseases[6,7]. The original BCG Pasteur strain was developed by 230 serial passages in liquid culture and has never been shown to revert to virulence in animals, indicating that the attenuating mutations in BCG are stable deletions and/or multiple mutations which do not readily revert. It is clear that BCG and other mycobacteria have potent nonspecific immunostimulatory properties, and preparations that contain mycobacterial components are widely used as adjuvants. While physiological differences

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between BCG and *M. tuberculosis* and *M. bovis* have been noted[8,9], the attenuating mutations which arose during serial passage of the original BCG strain have never been identified.

In recent reports, many investigators have described methods for using nucleic acid sequences to identify various specific members of the genus Mycobacterium[10~12]. Our interest in nucleic acid-based probe diagnoses for infectious diseases led us to examine members of the genus Mycobacterium for unique rRNA sequences that can be used to identify vaccines. In this paper, we report the cloning and analysis of *Mycobacterium bovis* BCG DNA using the PCR technique. In addition, the results of this study suggest that the fast-growing mycobacteria such as *M. smegmatis*, and slow-growing *M. bovis* BCG are significantly different in term of detection through genomic amplification.

MATERIALS AND METHODS

I. *Bacterial Strains*

The bacterial strains used in this study, including: BCG Brazilian, BCG Connaught, BCG Danish, BCG Glaxo, BCG Japanese, BCG Pasteur, BCG Russian, BCG Taiwan (Tokyo 172) and BCG Tice, are listed in Table 1. The BCG Taiwan strain was obtained from National Institute of Preventive Medicine.

Table 1. Mycobacterial strains used in the present study

Mycobacterium species	Source or description	
M. bovis BCG	Brazillian strain	ATCC35736[a]
M. bovis BCG	*Connaught strain*	ATCC35745
M. bovis BCG	*Danish strain*	ATCC35733
M. bovis BCG	*Glaxo strain*	ATCC35741
M. bovis BCG	*Japanese strain*	ATCC35737
M. bovis BCG	*Pasteur strain*	ATCC35734
M. bovis BCG	*Russian strain*	ATCC35740
M. bovis BCG	*Taiwan strain*	----------
M. bovis BCG	*Tice strain*	ATCC35743
M. smegmatis	----------	ATCC607

[a] American Type Culture Collection, Rockville, MD.

II. *Media and Reagents*

M. smegmatis and BCG cultures were grown in Middlebrook 7H9 medium (Difco) supplemented with albumin-dextrose complex (ADC; Difco) and 0.05% Tween 80 (M-ADC-TW browth) at 37°C in an incubator. Middlebrook 7H11(Difco), supplemented with oleic acid-ADC (OADC; Difco), was used as an agar medium. BCG cultures were grown for 3-4 weeks on agar medium followed by 2-3 weeks in broth.

III. *Specimen Processing for PCR*

Growths of BCG were harvested by means 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[13]. The pellet was then resuspended in distilled water and lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 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 . Subsequently, 5 mM NaCl and 10% acetyltrimethylammonium bromide/0.7 mM NaCl were mix added and kept at 65°C for 10 min. 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 Procedures*

The primers, BCGF1, BCGF2, BCGR1, BCGR2 used in this study are shown in Table 2. Each PCR mix contained 10 mM Tris (pH8.3), 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 polymerse; Perkin-Elmer Cetus, Norwalk, Conn.), and approximately 100 ng of genomic BCG DNA as template in a

Table 2. Oligonucleotide primers used in this study

Primer	Target	Sequence (5'-3')	Ref
BCGF1	16S-23S spacer	GAAGTCGTAACAAGGTAGCCG	1
BCGR1	16S-23S spacer	GCGTAGGCGTCGGTGACAAA	1
BCGF2	IS986	CGTGAGGGCATCGAGGTGGC	2
BCGR2	IS986	GCGTAGGCGTCGGTGACAAA	2

1. designed in this study
2. Hermans *et al.* [21]

thermal cycler (Perkin Elmer-Cetus, 9600). The amplifications were performed on 50 μL of sample solution, and 30 cycles were run for 1 min at 95°C, 30s at 55°C, and 1 min and 30s at 72°C, with a final elongation step of 10 min at 72°C. The PCR product, 10 μL, was fractionated on agarose electrophoresised, and visualized using ethidium bromide (Sigma Chemica Co., ST. Louis, Mo.). DNA bands with the expected size indicated positive reactions. Negative controls containing all PCR reagents except DNA were run in parallel with the samples. Recommended precautions were taken to avoid DNA contamination[14].

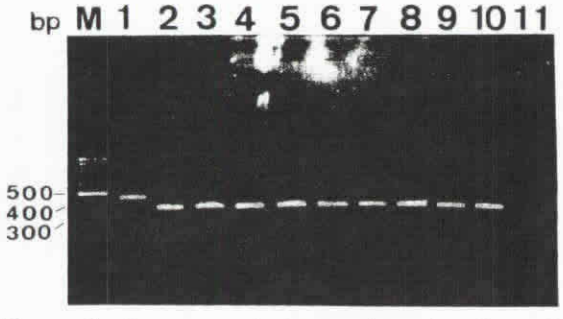

Figure 1. Agarose gel electrophoresis of amplified DNAs of different mycobacterial BCG strains by using oligonucleotide primers BCGF1 and BCGR1 in PCR. Lanel, *Mycobacterium smegmatis*; Lane 2, BCG Brazillian strain; Lane 3, BCG Connaught strain; Lane 4, BCG Danish Strain; Lane 5, BCG Glaxo strain; Lane 6, BCG Japanese strain; Lane 7, BCG Pasteur strain; Lane 8, BCG Russian strain; Lane 9, BCG Taiwan strain; Lane 10, BCG Tice strain; Lane 11, Negative control with water; M, 100 bp DNA ladder maker. Numbers on the left indicate sizes of standard DNA fragments in base pairs.

V. *Sequencing*

PCR products were sequenced directly using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) as Frothingham, *et al.* described[4]. The PCR primers were used as sequencing primers. Sequences from the two strands were combined; discrepancies were resolved through examination of chromatographs generated by the 373A DNA sequencer (Applied Biosystem).

RESULTS AND DISCUSSION

I. *DNA Amplification of BCG Vaccine*

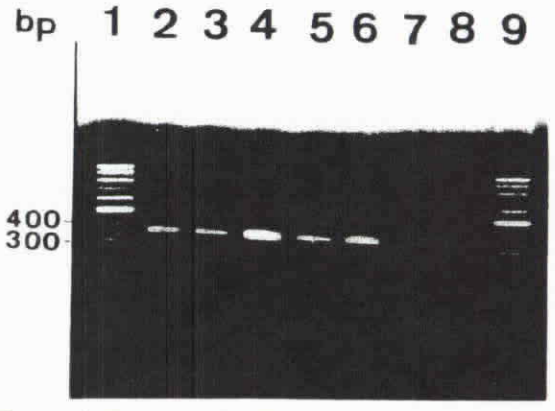

Figure 2. Results of PCR amplification of the *M. bovis* BCG 16S-23S spacer in different annealing temperature. template DNA: *M. bovis* BCG Taiwan strain. Lane 2 to 7 are: 40°C, 45°C, 50°C, 55°C, 60°C, 65°C by using oligonucleotide primers BCGF1 and BCGR1. Lane 1 and 9 are 100 bp DNA ladder markers. Lane 8, negative control with water. Numbers on the left indicate sizes of standard DNA fragments in base pairs.

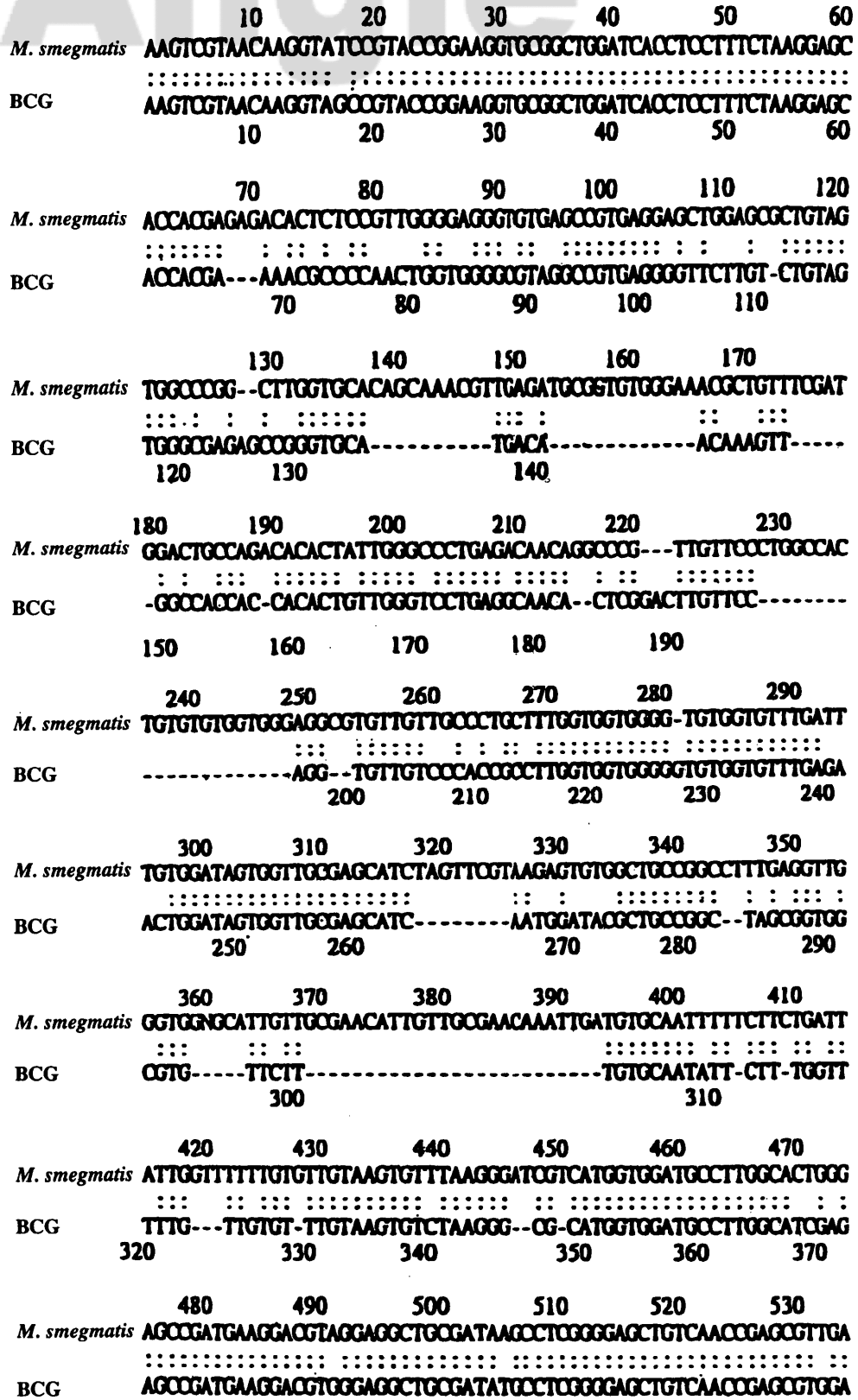

Figure 3. Nucleotide sequence of the spacer region between 16S and 23S rRNA genes. The noncoding strand of the *M. bovis* BCG (BCG) spacer region is shown together with that of *M. smegmatis* for comparison. Dots indicate nucleotides identical with those of *M. bovis* BCG. Bars indicate deletions.

Based on the sequence of 16S-23S rRNA intergenic spacer regions[15,16], two oligonucleotides, BCGF1 and BCGR1, were designed and synthesized. These oligonucleotides were used as primers for amplification of chromosomal DNA by PCR. As illustrated in Fig.1, after amplification of chromosomal DNAs of the *M. bovis* BCG strains, including Brazilian, Connaught, Danish, Japanese, Glaxo, Pasteur strain, Russian, Taiwan and Tice strains, were found to contain the 350-bp amplifiable fragment. *M. smegmatis* was tested similarly, and found to contain a 450 bp product but no 350 bp fragment. Thus, PCR consistently demonstrated a positive signal in all the 9 strains of *M. bovis* BCG but not in *M. smegmatis*. With all the different combinations of primers used in the PCR, the results were the same as above. Variation of the annealing temperature from 40°C to 65°C resulted in some visible PCR products (Fig.2). Because of the very high stringency of the annealing conditions, it is likely that there was virtually complete correspondence between primer and gene for the IS986 positive group. Under moderate stringency with an annealing temperatures of 50°C and 60°C, much more DNA was obtained. The specific DNA fragments, 350 bp, were amplified to a small extent when the annealing temperature under high stringency was 40~45°C and 65°C. It seems that using 50 or 55°C for the amplification reaction led to an optimal result. No specific PCR products were obtained in the control reaction mixture containing water.

II. *Identity with IS986*

All *M. bovis* BCG isolates are likely to contain a single IS986 copy at one particular location in the chromosome. This suggests that IS986 lost its transpostion capability before or during the 230 passages of the parental strain of *M. bovis* BCG, and that all the substrains are identical with respect to the site of insertion of IS986 in the chromosome (data not shown). This unique location in all *M. bovis* BCG strains will be useful in distinguishing BCG from other mycobacterial strains. Cousins e*t al.* used DNA amplification of a region of the insertion sequence by means of PCR for rapid identification of *M. bovis* and *M. tuberculosis*[16]. The amplification reaction using BCGF2 and BCGR2 as primer, produced a single 986 bp product, which was readily detected.

III. *Comparison of Sequences*

In this study, we used genomic amplification to identify BCG and the *M. smegmatis* strain. By this approach, three genomic regions of BCG and *M. smegmatis* were identified, cloned, and sequenced. The results of this study suggest that the fast-growing mycobacterium, *M. smegmatis* [17], and slow-growing *M. bovis* are significantly different in terms of detection through genomic amplification. Complete sequence comparisons of each amplified region from the *M. bovis* BCG strain and *M. smegmatis* revealed the precise junctions for each region of difference. (Fig.3). The amplified region from Connaught, Tice and Taiwan substrains of BCG showed almost the same DNA sequence. Examing the overall sequences of Connaught, Tice and Taiwan substrains of BCG, we found that among these three strains the highly conserved regions were found in 16S-23S spacer regions. These three isolates have high DNA homology and have highly conserved DNA sequences in several gene regions: the 16S rDNA, the 16S-to-23S rDNA internal transcribed spacer, and the dnaJ[18~20]. This has limited the use of sequence analysis for strain differentiation. The complete nucleotide sequence of the 16S-23S rRNA sapcer region gene of *M. bovis* BCG was determined. Its coding region was estimated to be 385 base pair, while *M. smegmatis* was 286 bp long. Another interesting point we have to stress regarding the spacer region is that *M. bovis* BCG and *M. smegmatis* have strikingly lower homology (66%). Overall, our data indicate that the 16S-23S spacer region is present in *M. bovis* BCG and is quite different in the *M. smegmatis* strain. The results of this study showed that no polymorphism was observed among BCG substrains suggests that the 16S-23S spacer is a relatively stable region which varies only occasionally. We will further investigate whether there is a correlation between the loss of virulence of *M. bovis* BCG

and the difference in the 16S-23S space in the chromosome.

REFERENCES

1. Broom, B. R. and Murray, J. L. 1992. Tuberculosis: Commentary on a reemergent killer. Science 257: 1055-1064.
2. Kochi, A. 1991. The global tuberculosis situation and the new control strategy of the World Health Organization. Tubercle 72: 1-6.
3. Raviglione, M. C., Snider, D. E. J. and Kochi, A. 1995. Global epidemeology of tuberculosis -Morbidity and mortality of a worldwide epidemic. JAMA. 273: 220-226.
4. Frothingham, R., Hills, H. G. and Wilson, K. H. 1994. Extensive DNA sequence conservation throughout the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. 32: 1639-1643.
5. Colditz, G. A., Brewer, T. F., Berkey, C. S., Wilson, M. E., Burdick, E., Fineberg, H.V. and Mosteller, F. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. JAMA. 271: 698-702.
6. Stover, C. K., Bansal, G. P., Hanson, M. S., Burlein, J. E., Palaszynski, S. R., Young, J. F., Koenig, S., Young, D.B., Sadziene, A. and Barbour, A. G. 1992. Protective immunity elicited by recombinant BCG expressing OspA lipoprotein: A candidate Lyme disease vaccine. J. Exp. Med. 178: 197-209.
7. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T. R. G., Bansal, G.P., Young, J. F., Lee, M. H., Hatfull, G. F. 1991. New use of BCG for recombinant vaccines. Nature (London) 351: 456-460.
8. Strain, S. M., Toubiana, R., Ribi, E. and Parker, R. 1977. Separation of the mixture of trehalose 6,6'-dimyolates comprising the *mycobacterial* glycolipid fraction, "P3". Biochem. Biophys. Res. Commun. 77: 449-456.
9. Takayama, K., Wang, L. and David, H. L. 1972. Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 2: 29-35.
10. Boddinghaus, B., Rogall, T., Flohr, T., Blocker, H. and Bottger, E.C. 1990. Detection and identification of *mycobacteria* by amplification of rRNA. J. Clin. Microbiol. 28: 1751-1759.
11. Hughes, M. S., Skuce, R. A., Beck, L. A. and Neill, S. D. 1993. Identification of mycobacteria from animals by restriction enzyme analysis and direct DNA cycle sequencing of polymerase chain reaction-amplified 16S rRNA gene sequences. J. Clin. Microbiol. 31: 3216-3222.
12. Teleni, A., Marchesi, F., Balz, M., Bally, F., Bottger, E. C. and Bodmer, T. 1993. Rapid identification of mycobacterial to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31: 175-178.
13. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual. 2nd ed. pp. E5-E6. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., U.S.A.
14. de Wit, D., Steyn, L., Shoemaker, S. and Sogin, M. 1990. Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. J. Clin. Microbiol. 28: 2437-2441.
15. Nakagawa, T., Uemori, T., Asada, K., Kato, I. and Harasawa, R. 1992. Acholeplasma laidlawii has tRNA genes in the 16S-23S spacer of the rRNA operon. J. Bacteriol. 174: 8163-8165.
16. Cousins, D. V., Wilton, S. D. and Francis, B. R. 1991. Use of DNA amplification for the rapid identification of *Mycobacterium bovis* Vet. Microbiol. 27: 187-195.
17. Kolk, A. H., Noordhoek, G. T., de Leeuw, O., Kuijper, S. and van Embden, J. D. A. 1994. *Mycobacterium smegmatis* strain for detection of *Mycobacterium tuberculosis* by PCR used as internal control for inhibition of amplification and for quantification of bacteria. J. Clin. Microbiol. 32: 1354-1356.

18. Frothingham, R. and Wilson, K. H. 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J. Bacteriol. 175: 2818-2825.
19. Kirschner, P., Springer, B., Bogel, U., Meier, A., Wrede, A., Kiekenbeck, M., Bange, F. C. and Bottger, E. C., 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: Report of a 2-year experience in a clinical laboratory. J. Clin. Microbiol. 31: 2882-2889.
20. Takewaki, S. L., Okuzumi, K., Manabe, I., Tanimura, M., Miyamura, K., Nakahara, K. I., Yazaki, Y., Ohkubo,. A. and Nagai, R. 1994. Nucleotide seqnence comparison of the mycobacterial dnaJ gene and PCR-restriction fragment length polymorphism analysis for identification of mycobacterial species. Int. J. Syst. Bacteriol. 44: 159-166.
21. Hermans, P. W. H., van Soolingen, D., Dale, J. W., Schuitema, A. R., McAdam, R. A., Catty, D. and van Embden, J. D. A. 1990. Insertion element IS986 from *Mycobacterium tuberculosis*: A useful tool for diagnosis and epidemiology of tuberculosis. J. Clin. Microbiol. 28: 2051-2058.

核酸增幅反應在BCG疫苗與*M. smegmatis*之鑑別

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摘 要

本研究利用16S-23S rRNA spacer基因段鑑別BCG疫苗株，包括：Brazilian, Connaught, Danish, Glaxo Japanese, Pasteur, Russian, Taiwan和Tice等9株及另一非疫苗株*Mycobacterium smegamatis*。使用BCGF1和BCG R1作爲引子對。結果發現經增幅之PCR產物在疫苗株產生350 bp，對於非疫苗株則產生約456 bp。由本研究所設計之引子對，具快速簡便及有效偵測病原菌之特性並可用於疫苗株菌分離之鑑定，將可嘗試發展爲基因探針作爲鑑定之工具。

關鍵詞：BCG疫苗，*M. smegmatis*，核酸增幅反應。