Journal of Clinical Microbiology, Apr. 1997, p. 965–968 Vol. 35, No. 4
0095-1137/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Genetic Identification of *Mycobacterium bovis* BCG by Restriction Fragment Length Polymorphism Analysis of the Direct-Repeat Region

S. T. HOWARD,[1]* A. LASZLO,[2] AND W. M. JOHNSON[1]

Microbial Genetics Laboratory[1] and National Reference Centre for Tuberculosis,[2] National Laboratory for Bacteriology and Enteric Pathogens, Bureau of Microbiology, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada

Received 13 September 1996/Returned for modification 21 November 1996/Accepted 14 January 1997

Restriction fragment length polymorphism (RFLP) analysis was performed on the direct repeat (DR) regions of 14 strains of *Mycobacterium bovis* BCG. With *Alu*I-digested DNA, BCG Japanese, Russian, and Mexican had differing RFLP patterns but 11 strains, including Pasteur, Glaxo, and Tice, had an identical pattern not detected in over 60 other strains of the *M. tuberculosis* complex. DR analysis can aid in confirming the identification of clinical BCG isolates.

Vaccination of newborns and children with *Mycobacterium bovis* BCG has been part of tuberculosis control programs in both developing and industrialized countries and is still recommended for children at high risk of infection with *M. tuberculosis* (22, 23). BCG is also used in immunotherapy for certain types of cancer, particularly bladder carcinoma (3, 19, 20). Complications such as osteitis, meningitis, and sepsis can occasionally result from the administration of BCG (3, 12, 15, 24), and acid-fast bacilli isolated from affected patients must be correctly identified so that appropriate treatment can be given.

M. bovis BCG was originally derived 75 years ago by serial passage of *M. bovis*, a member of the *M. tuberculosis* complex (18, 23). Because members of the complex are very closely related, identifying a clinical isolate as BCG by genetic means can be difficult, and no single target has been found which yields unambiguous results. Strains of BCG carry only one or two copies of the insertion sequence IS*6110* (7, 9, 10), a mobile genetic element which has been found exclusively in the *M. tuberculosis* complex (10). Strains of *M. tuberculosis* have been detected, though, which also have low copy numbers of this element (5, 11, 21). The presence of the insertion element IS*1081* in an 8-kbp *Pvu*II fragment has been used to identify BCG (24, 27), but Huh and coworkers (13) discovered a group of *M. tuberculosis* strains from Korea that contained IS*1081* in similarly sized fragments, although these strains differed from BCG in having multiple copies of IS*6110*. Targeting IS*1081* or IS*6110* has therefore proven to be less useful for identifying BCG than other methods, such as mycolic acid analysis (6), PCR (8), or DNA restriction fragment analysis (4, 29).

Members of the *M. tuberculosis* complex have a genomic region that consists of multiple copies of a 36-bp element, the direct repeat (DR), interspersed with short stretches of unique sequence (9). Hermans and coworkers (9) examined several strains of BCG by Southern blotting of DNA digested with the restriction enzyme *Pvu*II and found identical but unremarkable restriction fragment length polymorphism (RFLP) patterns with the DR element as a probe. We examined 14 strains of BCG by using the restriction enzyme *Alu*I, which produces more discriminatory patterns in the DR region (26), and found that most BCG strains shared a distinctive RFLP pattern.

We obtained a strain of the *M. tuberculosis* complex that had been isolated from the urine of a patient treated for bladder carcinoma. Conventional identification procedures (14) indicated that the isolate (strain A) was *M. bovis* BCG. For preparation of DNA from strain A, the culture was grown on Lowenstein-Jensen slants for 3 weeks. The method used for extraction of DNA was based on a published procedure (28) using the denaturant *N*-cetyl-*N*,*N*,*N*,-trimethylammonium bromide (CTAB) after protease treatment of cells. RFLP analysis of the DNA by a standardized method (25) determined that strain A had a single copy of IS*6110* present in a 1.8-kbp *Pvu*II fragment (data not shown). For analysis of the DR region, 2 μg of DNA was digested with 5 U of *Alu*I (Boehringer Mannheim, Laval, Quebec, Canada) for 4 h at 37°C. DNA extracts from clinical isolates of *M. tuberculosis* with one or two copies of IS*6110* were also digested with *Alu*I. After digestion, DNA samples were mixed with 10 ng of 100-bp ladder (Gibco-BRL) in loading buffer and then electrophoresed on a 1.5% agarose gel overnight at 35 V in Tris-acetate buffer. The DNA was transferred onto a nylon membrane by Southern blotting with a Stratagene Posiblot pressure blotter (Stratagene, La Jolla, Calif.), and the membrane was probed with 400 ng of the 36-base oligonucleotide DR-r (9). Oligonucleotide labelling, hybridization, and detection steps were carried out with a 3′ oligolabelling system (Amersham, Oakville, Ontario, Canada) according to the manufacturer's instructions. Fragment sizes were determined by probing separately for 100-bp ladder and superimposing the autoradiographic images.

RFLP analysis of the DR region (Fig. 1) revealed that strain A (lane 9) had a unique pattern characterized by a triplet of fragments, each approximately 800 bp in size. This RFLP pattern was distinct from those of nine strains of *M. tuberculosis* with low copy numbers of IS*6110* (Fig. 1, lanes 2 to 8, 10, and 11) and from those of 40 other clinical isolates of *M. tuberculosis* (data not shown). Next, we analyzed 14 strains of *M. bovis* BCG and found that 11 had the same pattern as, or patterns highly similar to, that of strain A (Table 1). All of these 11 strains, several of which have RFLP patterns shown in Fig. 2, had the distinctive triplet at 800 bp. The patterns of the Pasteur (Fig. 3, lane 10) and Glaxo (data not shown) strains, which are two of the most frequently used vaccine strains (12), were identical to that of strain A. Although the identity of strain A

* Corresponding author. Mailing address: Bureau of Microbiology, Laboratory Centre for Disease Control, Postal Locator 0702E3, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada. Phone: (613) 941-2035. Fax: (613) 941-2408. E-mail: susan_howard_at_ncot221@isdtcp3.hwc.ca.

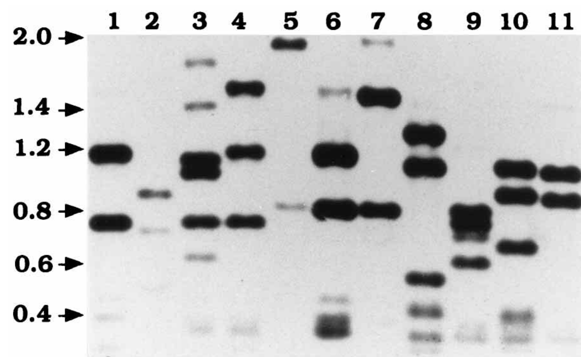

FIG. 1. DR RFLP analysis of *M. tuberculosis* complex strains with low copy numbers of IS*6110*. Lane 1, H37Rv (reference strain); lanes 2 to 8, 10, and 11, clinical isolates of *M. tuberculosis*; lane 9, clinical isolate of BCG (strain A). Arrows indicate positions of molecular size markers (sizes are in kilobase pairs).

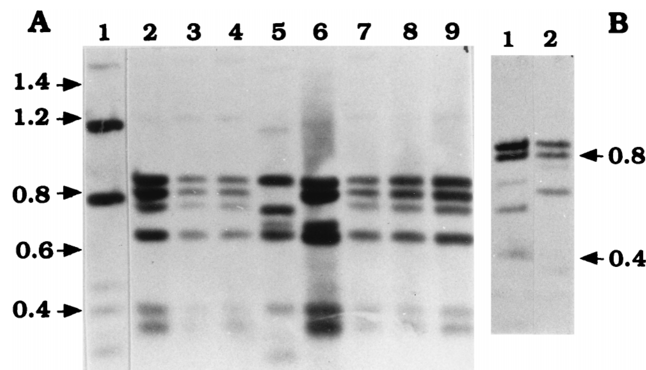

FIG. 2. DR RFLP analysis of *M. bovis* BCG strains. Lanes (strains): (A) 1, H37Ra (reference strain); 2, Montreal; 3, Birkhaug; 4, Swedish; 5, Mexican; 6, Russian; 7, Australian; 8, Tice; 9, Montreal (isoniazid resistant); (B) 1, Japanese; 2, Pasteur (streptomycin resistant). Arrows indicate positions of molecular size markers (sizes are in kilobase pairs).

is unknown, it is possibly either BCG Tice (Fig. 2A, lane 8) or Pasteur (Fig. 3), both of which are used in cancer immunotherapy (3, 19) and, like strain A, have only one copy of IS*6110* (5).

Minor variations of the strain A pattern were found in BCG Brazilian (data not shown) and in a streptomycin-resistant variant of the Pasteur strain (Fig. 2B, lane 2). The weak bands present at 1.2 and 1.6 kbp in the typical BCG pattern (Fig. 2A, lanes 2 to 4 and 7 to 9) were not detected in these two strains, but repeat analyses of strain A and other BCG strains revealed that these two fragments were not always clearly visible. The remaining bands in the RFLP pattern were, therefore, considered more reliable for establishing identity to strain A. More significant variations in the DR region were detected in the Russian, Mexican, and Japanese strains of BCG (Fig. 2). The smallest fragment of the 800-bp triplet was absent in the Russian strain (Fig. 2A, lane 6), and instead there was a smaller fragment of approximately 700 bp. Changes in the triplet and additional differences occurred in the Mexican (Fig. 2A, lane 5) and Japanese (Fig. 2B, lane 1) strains.

TABLE 1. Strains of *Mycobacterium tuberculosis* complex used in study

Strain	ATCC[a] no.	Strain A pattern[b]
M. bovis BCG		
Birkhaug	35731	+
Swedish	35732	+
Danish (BCG Copenhagen)	35733	+
Pasteur	35734	+
Montreal	35735	+
Brazilian	35736	+
Japanese (BCG Tokyo?)	35737	−
Mexican	35738	−
Australian	35739	+
Russian	35740	−
Glaxo	35741	+
Tice	35743	+
Montreal (isoniazid resistant)	35747	+
Pasteur (streptomycin resistant)	35748	+
M. africanum	35711	−
M. microti	19422	−
M. tuberculosis		
Clinical isolates		−
H37Rv	27294	−
H37Ra	25177	−
M. bovis		
Strains from ATCC and ADRC[c]		−

[a] ATCC, American Type Culture Collection.
[b] Strain A DR RFLP pattern present, +; pattern not present, −.
[c] ADRC, Animal Disease Research Centre, Rijks Institute, Bilthoven, The Netherlands.

Because of the close relationship of *M. bovis* and BCG, we examined the DR regions of 15 strains of *M. bovis* by RFLP, and several of the resultant patterns are shown in Fig. 3. Some *M. bovis* strains (see, for example, lanes 5 to 7) had RFLP patterns with greater resemblance to the standard BCG pattern (lane 10) than had any of the *M. tuberculosis* strains examined (Fig. 1 and data not shown), but none had the identical pattern. Two other members of the *M. tuberculosis* complex, *M. africanum* and *M. microti*, also exhibited RFLP patterns which were distinguishable from that of BCG Pasteur (Fig. 3, lanes 10 to 12).

The original strain of *M. bovis* BCG developed by Calmette and Guérin was distributed to laboratories in several countries (18, 23), and different passage histories of the cultures resulted in daughter strains with different properties (2, 7, 16–18). Some of the relationships among BCG strains suggested by RFLP

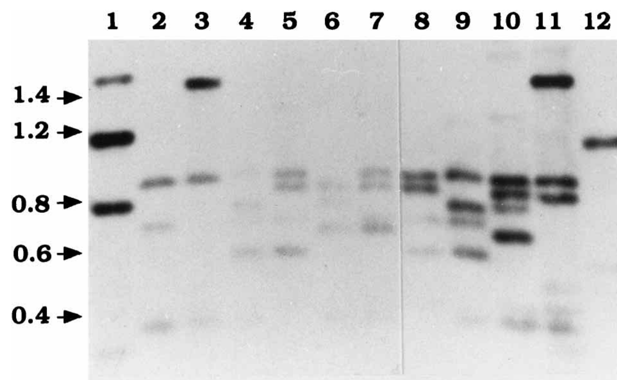

FIG. 3. DR RFLP of *M. tuberculosis* complex strains. Lane 1, H37Rv (reference strain); lanes 2 to 9, *M. bovis*; lane 10, BCG Pasteur; lane 11, *M. africanum*; lane 12, *M. microti*. Arrows indicate positions of molecular size markers (sizes are in kilobase pairs).

analysis are supported by studies on the gene encoding the protein MPB64 (16). This gene is absent in the Copenhagen, Pasteur, Glaxo, and Tice strains, all of which have the strain A pattern, but it is present in BCG Russian, which has a different RFLP pattern (Fig. 2A, lane 6). Li and coworkers (16) also detected MPB64 in BCG Tokyo; although different terminologies are used, BCG Tokyo and BCG Japan appear to correspond to the BCG Japanese that we used (7, 18). BCG Japanese and Russian also differ from the other four strains in that they produce high levels of the protein MPB70 (1, 2, 17).

Fomukong and coworkers (7) noted that a subset of BCG strains that includes the Japanese and Russian strains has properties which more closely resemble those of *M. bovis*. In light of this, it is interesting that two related strains of *M. bovis* (ATCC 35723 and 35729) (Fig. 3, lanes 5 and 8) have DR RFLP patterns markedly similar to that of BCG Japanese (Fig. 2B, lane 1). However, BCG Swedish and Brazilian were also included in the subset, and their RFLP patterns match that of strain A. BCG Swedish and Brazilian might be more closely related to the majority of BCG strains than are BCG Japanese and Russian, but it is also conceivable that the similarity of their DR regions to the strain A pattern could have arisen independently, particularly as the number of genetic changes required to generate the observed polymorphism is not known. In contrast to the results of the DR analysis, BCG Swedish was found to be more similar to BCG Japanese than to BCG Copenhagen by restriction fragment analysis (4). It is apparent that additional genetic analysis will be required to determine the degree of divergence that has occurred between different BCG strains and between different laboratory stocks of the same strain.

The similarity of the DR regions of some *M. bovis* and *M. bovis* BCG strains supports the findings of Zhang and coworkers (29). They observed that the pulsed-field gel patterns of *M. bovis* and BCG strains exhibited much greater similarity to each other than to patterns from *M. tuberculosis* strains. However, although they were able to distinguish between most BCG strains through the use of several enzymes, an examination of their results revealed no correlation between BCG strains with DR pattern A and any of the groupings based on patterns of large restriction fragments.

DR RFLP patterns appear to be more stable than IS*6110*-based patterns. For example, the *M. tuberculosis* reference strain H37Rv (Fig. 1, lane 1) and its attenuated variant, H37Ra (Fig. 2A, lane 1), have identical RFLP patterns in the DR region, whereas their IS*6110*-based patterns show several differences (11, 26). We expect, therefore, that the DR region will be a useful target for identifying clinical isolates of BCG despite the existence of variation in a few BCG strains. The standard (strain A) pattern was not detected in over 65 other isolates of the *M. tuberculosis* complex. However, strains of *M. tuberculosis* or *M. bovis* may be discovered which have the standard BCG pattern, so we recommend that both genetic and biochemical analyses be performed to confirm the identification of BCG isolates obtained from patients.

When examining the DR region, we have observed some variation in band intensity which does not occur with IS*6110* analysis. In IS*6110* analysis, each detected *Pvu*II fragment contains only one copy of the insertion element, whereas in DR analysis, some restriction fragments contain multiple copies of the DR and so will be detected more easily than fragments containing only a few copies. DR analysis may therefore be more susceptible to minor changes in procedure which affect band intensity. A standardized protocol, such as that developed for IS*6110*-based RFLP analysis (25), may be warranted to ensure accurate comparison of DR-based RFLP patterns between laboratories.

We thank Vera Handzel, Nicole Pearson, Matthew Oughton, and Robert Vogrig for their technical assistance.

S.T.H. was supported by a Canadian Bacterial Disease Network fellowship.

REFERENCES

1. **Abou-Zeid, C., M. Harboe, and G. A. W. Rook.** 1987. Characterization of the secreted antigens of *Mycobacterium bovis* BCG: comparison of the 46-kilodalton dimeric protein with proteins MPB64 and MPB70. Infect. Immun. **55:**3213–3214.
2. **Abou-Zeid, C., I. Smith, J. Grange, J. Steele, and G. Rook.** 1986. Subdivision of daughter strains of bacille Calmette-Guérin (BCG) according to secreted protein patterns. J. Gen. Microbiol. **132:**3047–3053.
3. **Brosman, S. A., and D. L. Lamm.** 1990. The preparation, handling and use of intravesical bacillus Calmette-Guérin for the management of stage Ta, T1, carcinoma in situ and transitional cell cancer. J. Urol. **144:**313–315.
4. **Collins, D. M., and G. W. De Lisle.** 1987. BCG identification by DNA restriction fragment patterns. J. Gen. Microbiol. **133:**1431–1434.
5. **Das, S., C. N. Paramasivan, D. B. Lowrie, R. Prabhakar, and P. R. Narayanan.** 1995. IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, South India. Tubercle Lung Dis. **76:**550–554.
6. **Floyd, M. M., V. A. Silcox, W. D. Jones, Jr., W. R. Butler, and J. O. Kilburn.** 1992. Separation of *Mycobacterium bovis* BCG from *Mycobacterium tuberculosis* and *Mycobacterium bovis* by using high-performance liquid chromatography of mycolic acids. J. Clin. Microbiol. **30:**1327–1330.
7. **Fomukong, N. G., J. W. Dale, T. W. Osborn, and J. M. Grange.** 1992. Use of gene probes based on the insertion sequence IS*986* to differentiate between BCG vaccine strains. J. Appl. Bacteriol. **72:**126–133.
8. **Frothingham, R.** 1995. Differentiation of strains in *Mycobacterium tuberculosis* complex by DNA sequence polymorphisms, including rapid identification of *M. bovis* BCG. J. Clin. Microbiol. **33:**840–844.
9. **Hermans, P. W. M., D. van Soolingen, E. M. Bik, P. E. W. de Haas, J. W. Dale, and J. D. A. van Embden.** 1991. The insertion element IS*987* from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infect. Immun. **59:**2695–2705.
10. **Hermans, P. W. M., D. van Soolingen, J. W. Dale, A. R. J. Schuitema, R. A. McAdam, D. Catty, and J. D. A. van Embden.** 1990. Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. J. Clin. Microbiol. **28:**2051–2058.
11. **Howard, S. T.** Unpublished data.
12. **Huebner, R. E., and G. W. Comstock.** 1994. BCG vaccine, p. 335–348. *In* L. N. Friedman (ed.), Tuberculosis: current concepts and treatment. CRC Press, Inc., Boca Raton, Fla.
13. **Huh, Y. J., D. I. Ahn, and S. J. Kim.** 1995. Limited variation of DNA fingerprints (IS*6110* and IS*1081*) in Korean strains of *Mycobacterium tuberculosis*. Tubercle Lung Dis. **76:**324–329.
14. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology, a guide for the level III laboratory. Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, Ga.
15. **Kröger, L., M. Korppi, E. Brander, H. Kröger, O. Wasz-Höckert, A. Backman, J. Rapola, K. Launiala, and M.-L. Katila.** 1995. Osteitis caused by bacille Calmette-Guérin vaccination: a retrospective analysis of 222 cases. J. Infect. Dis. **172:**574–576.
16. **Li, H., J. C. Ulstrup, T. Ø. Jonassen, K. Melby, S. Nagai, and M. Harboe.** 1993. Evidence for absence of the MPB64 gene in some substrains of *Mycobacterium bovis* BCG. Infect. Immun. **61:**1730–1734.
17. **Miura, K., S. Nagai, M. Kinomoto, S. Haga, and T. Tokunaga.** 1983. Comparative studies with various substrains of *Mycobacterium bovis* BCG on the production of an antigenic protein, MPB70. Infect. Immun. **39:**540–545.
18. **Osborn, T. W.** 1983. Changes in BCG strains. Tubercle **64:**1–13.
19. **Pansadoro, V., P. Emiliozzi, L. Defidio, D. Donadio, A. Florio, S. Maurelli, S. Lauretti, and C. N. Sternberg.** 1995. Bacillus Calmette-Guérin in the treatment of stage T1 grade 3 transitional cell carcinoma of the bladder: long-term results. J. Urol. **154:**2054–2058.
20. **Rosenthal, S. R.** 1980. BCG in cancer and leukemia, p. 295–324. *In* S. R. Rosenthal (ed.), BCG vaccine: tuberculosis—cancer. PSG Publishing Company, Inc., Littleton, Mass.
21. **Small, P. M., P. C. Hopewell, S. P. Singh, A. Paz, J. Parsonnet, D. C. Ruston, G. F. Schecter, C. L. Daley, and G. K. Schoolnik.** 1994. The epidemiology of tuberculosis in San Francisco: a population-based study using conventional and molecular methods. N. Engl. J. Med. **330:**1703–1709.
22. **Speert, D. P.** 1995. The use of bacille Calmette-Guérin vaccination in children. Can. J. Infect. Dis. **6:**232–233.
23. **ten Dam, H. G.** 1995. BCG vaccination: an old idea revisited, p. 109–128. *In* M. D. Rossman and R. R. MacGregor (ed.), Tuberculosis: clinical management and new challenges. McGraw-Hill, Inc., New York, N.Y.

24. **van Deutekom, H., Y. M. Smulders, K. J. Roozendaal, and D. van Soolingen.** 1996. Bacille Calmette-Guérin (BCG) meningitis in an AIDS patient 12 years after vaccination with BCG. Clin. Infect. Dis. **22:**870–871.
25. **van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
26. **van Soolingen, D., P. E. W. de Haas, P. W. M. Hermans, P. M. A. Groenen, and J. D. A. van Embden.** 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **31:**1987–1995.
27. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, and J. D. A. van Embden.** 1992. Insertion element IS*1081*-associated restriction fragment length polymorphisms in *Mycobacterium tuberculosis* complex species: a reliable tool for recognizing *Mycobacterium bovis* BCG. J. Clin. Microbiol. **30:**1772–1777.
28. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, D. R. Soll, and J. D. A. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
29. **Zhang, Y., R. J. Wallace, and G. H. Mazurek.** 1995. Genetic differences between BCG substrains. Tubercle Lung Dis. **76:**43–50.