Epidemiol. Infect. (2003), **130**, 485–490. © 2003 Cambridge University Press
DOI: 10.1017/S095026880300829X Printed in the United Kingdom

A novel multiplex-PCR for the rapid identification of *Mycobacterium bovis* in clinical isolates of both veterinary and human origin

L. COBOS-MARÍN[1], J. MONTES-VARGAS[2], S. RIVERA-GUTIERREZ[3], A. LICEA-NAVARRO[4], J. A. GONZÁLEZ-Y-MERCHAND[3] AND I. ESTRADA-GARCÍA[1]*

[1] *Departamento de Inmunologia, Escuela Nacional de Ciencias Biologicas, IPN; Prolongacion Carpio y Plan de Ayala s/n, Col. Sto. Tomas, Mexico D.F., 11340, Mexico*
[2] *CANETB, SAGARPA, Av. Del charro 770-1sur Cd Juarez, Chih. 32320, México*
[3] *Departamento de Microbiologia, Escuela Nacional de Ciencias Biologicas, IPN; Prolongacion Carpio y Plan de Ayala s/n, Col. Sto. Tomas, Mexico D.F., 11340, Mexico*
[4] *Departamento de Acuicultura y Biotecnología Marina. CICESE, Km 107 Carretera Tijuana-Ensenada, Ensenada, B.C., Mexico*

(*Accepted 27 December 2002*)

SUMMARY

Bovine tuberculosis is a zoonotic disease that not only causes huge economic losses but also poses an important risk for human infection. The definitive identification of a clinical isolate relies on time-consuming, highly specialized and laborious biochemical tests. We have developed a method for the rapid and reliable identification of *Mycobacterium bovis* and for its simultaneous differentiation from other members of the *M. tuberculosis* complex. Furthermore, the technique also allowed us to distinguish *M. tuberculosis* complex members from other *Mycobacterial* species. The method comprises both a single PCR and a multiplex-PCR and can be confidently applied to samples of both veterinary and human origin.

INTRODUCTION

Bovine tuberculosis is an infectious, chronic and progressive disease with a worldwide distribution, caused primarily by *M. bovis*. The presence of the disease in a herd is of the outmost relevance to public health since it could be a great risk of infection for human beings [1].

Even though the worldwide incidence of bovine tuberculosis has noticeably diminished in recent years, it remains a risk factor in many Latin American countries like Mexico and Argentina, where its prevalence has been calculated to be 2% and 4% respectively [2]. Even in developed countries such as England, the veterinary disease is prevalent. Indeed, there were 1031 infected herds and 9000 cases estimated during 2000, compared with 89 herds and 600 cases in 1979 [3].

Human tuberculosis caused by *M. bovis* is clinically indistinguishable from the disease caused by *M. tuberculosis*. Many cases occur in young people as a consequence of drinking contaminated milk. The incidence of tuberculosis in slaughterhouse and rural workers suggests that contact with cattle can lead to this disease [1, 4]. In Latin America it is estimated that 2% of human pulmonary tuberculosis and 8% of human non-pulmonary tuberculosis is caused by *M. bovis* [4]. However, in some areas of Mexico, this percentage has been calculated to be as high as 14% for human pulmonary tuberculosis [5].

Furthermore, some HIV patients have developed tuberculosis caused by either reactivation of a previous BCG vaccination [6], or non-tuberculous mycobacteria [7]. Accurate identification of the pathogen

* Author for correspondence.

has not only epidemiologic implications but is also relevant for the management of patients, given that bovine strains can be resistant to some antibiotics like pyrazinamide [1].

M. bovis is a member of the *M. tuberculosis* complex (MTC), which also includes *M. canetii*, *M. tuberculosis*, *M. africanum* and *M. microti* [8]. The routine identification of *M. bovis* after the bacteriological isolation is based on colony morphology and biochemical tests; however these tests are both time-consuming, and highly sophisticated [9].

Several molecular biology techniques have been explored in order to identify the pathogen rapidly with a high degree of sensitivity. Some of the tests that have been used to confirm the presence of *M. bovis* in clinical samples include RFLP analysis [10–13] or spoligotyping [14]. Most of these techniques, however, are not suitable for routine analysis in clinical laboratories.

In this work, a PCR technique was designed using primers of proven reliability [15–17]. The procedure allows the user to determine simultaneously whether a clinical isolate belongs to the *Mycobacterium* genus, to the *M. tuberculosis* complex and to the *M. bovis* species. A set of primers was used to amplify region of difference 4 (RD4), which is present in the genome of all members of the MTC except *M. bovis* [8, 15, 18]; these primers were combined with another set that amplified a region located within the 3′ end of the *murA* gene and a sequence within the 16 S rRNA gene; this fragment has been shown to be present in all mycobacteria [16, 19]. In parallel, another sample was analysed by PCR with a set of primers that specifically amplify a fragment containing the promoter region of the rRNA operon (*rrnA*) and the 5′ end of the 16 S rRNA gene; this fragment has been shown to be present only in members of the MTC [16, 20]. The products obtained by the use of the three sets of primers rapidly revealed both the presence of *M. bovis* in clinical isolates and its differentiation from the other MTC.

MATERIALS AND METHODS

Bacterial strains and media

M. bovis BCG Danish strain (provided by the Instituto Nacional de Higiene, Mexico) was used in this study because it is the main strain used for vaccination locally; other strains investigated were *M. bovis* AN5, *M. tuberculosis* H37Rv TMC102, *M. microti* NCTC OV254, *M. africanum* ATCC 25420, *M. canetii* 17727, *M. avium* TMC 716, *M. phlei* TMC 1516, *M. smegmatis* NCTC 8159 and *M. fortuitum* ATCC 6841. Strains were grown on Löwenstein–Jensen slopes. *Bacillus megaterium* ATCC 14581 and *B. subtilis* ATCC 6051 were grown on BHI medium (Difco Lab., Detroit, MI, USA). Fifty-eight clinical isolates identified as *M. bovis* by biochemical tests (niacin and nitrate negative) were included; these were obtained from the liver, lung and lymph nodes of cows with a tuberculous lesion and were grown on Stonebrink medium [21]. Sputum was collected from 120 patients suffering from tuberculosis. Each sample was cultured on Lowenstein–Jensen slopes and 97 samples developed growth. All strains were analysed by standard biochemical tests (niacin and nitrate). *M. bovis* was identified in three samples and confirmed by the gyrB PCR–RFLP testing [12]. *M. tuberculosis* was identified in the remaining samples and confirmed by the gyrB PCR–RFLP testing [12]. The 3 *M. bovis* isolates and 20 representative isolates from *M. tuberculosis* were selected for further study.

DNA isolation

A loopful of bacterial mass from solid cultures was resuspended in 0·5 ml of 10 mM Tris–HCl/1 mM EDTA/1% Triton X-100, and submitted to three cycles of freeze-boiling (5 min, −70 °C; 10 min, 100 °C) as described by Da Silva et al. [11]. *M. leprae* DNA isolated from armadillos (batch CD213) was provided by Dr M. J. Colston (NIMR, London, UK) and *Streptomyces coelicolor* DNA, ETHZ1174 (Institute des Eidgenossische Technischen Halschule, Switzerland) was provided by Dr C. Hernandez (Departamento de Microbiologia, Escuela Nacional de Ciencias Biologicas, IPN, Mexico).

DNA polymerase chain reaction (PCR)

Bacterial DNA (50–100 ng) was subjected to PCR using a standard *Taq* polymerase (Life Technologies, Rockville, MD, USA) in a total volume of 50 μl. One gene fragment was amplified using the primer combination of RAC1 (5′-TCGATGATCACCG-AGAACGTGTTC-3′) and RAC8 (5′-CACTGGTG-CCTCCCGTAGG-3′) coding for the last 99 codons of the *murA* gene, the promoter region of the *rrnA* operon and the 5′ end of the 16 S rRNA gene (Fig. 1*a*). The target for primer RAC1 is the sequence complementary to positions 46–69 of the 3′ end of

https://doi.org/10.1017/S095026880300829X Published online by Cambridge University Press

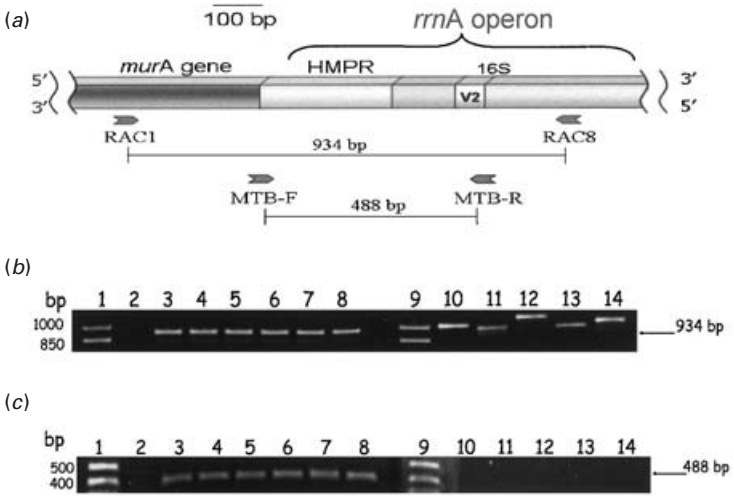

Fig. 1. Amplification by a PCR of genomic regions of different mycobacterial species. (*a*) Genomic DNA regions of the *M. tuberculosis* complex, amplified with the MTB-F/MTB-R and RAC1/RAC8 primers. ➡, primer binding sites; V2, variable region 2; HMPR, hypervariable multiple promoter region. (*b*) Products obtained by amplifying a fragments corresponding to the last 99 codons of the *murA* gene to position 357 of the 16 S rRNA gene (primers RAC1/RAC8). Lane 1, molecular-size markers; lane 2, no DNA; lane 3, *M. africanum*; lane 4, *M. bovis*; lane 5, *M. bovis* BCG; lane 6, *M. canetti*; lane 7, *M. microti*; lane 8, *M. tuberculosis* H37Rv; lane 9, molecular-size markers; lane 10, *M. avium*; lane 11, *M. leprae*; lane 12, *M. fortuitum*, lane 13, *M. phlei*; lane 14, *M. smegmatis*. (*c*) Products obtained by amplifying the region from the last five codons of *murA* gene, to the V2 region of the 16 S rRNA gene (primers MTB-F/MTB-R). Lane 1, molecular-size markers; lane 2, no DNA; lane 3, *M. africanum*; lane 4, *M. bovis*; lane 5, *M. bovis* BCG; lane 6, *M. canetti*; lane 7, *M. microti*; lane 8, *M. tuberculosis* H37Rv; lane 9, molecular-size markers; lane 10, *M. avium*; lane 11, *M. leprae*; lane 12, *M. fortuitum*; lane 13, *M. phlei*; lane 14, *M. smegmatis*.

murA gene of *M. tuberculosis* (EMBL Data Bank, accession number X87944; see also [19]). The target for RAC8 is positions 339–357 of the 16 S rRNA coding region [19]. Amplification was achieved using 1·5 mM $MgCl_2$, 200 μM dNTPs, 10% DMSO and 0·4 μM of primers RAC1/RAC8. The amplification procedure consisted of 36 cycles at 95 °C/30 s, 55 °C/1 min and 72 °C/2 min followed by a final extension step at 72 °C/5 min.

Another gene fragment coding for the last five codons of *murA* gene, the promoter region of the *rrnA* operon and the 5′ end of the 16 S rRNA gene was synthesized using the primer combination MTB-F (5′-CGGGTATGCTGTTAGGCGACG-3′) and MTB-R (5′-CCACCACAAGACATGCATG-3′) (see Fig. 1*a*). The target for primer MTB-F is the sequence complementary to the last 15 nucleotides of the *murA* gene and the first 6 nucleotides of the promoter region of the *rrnA* operon [16]. The target for primer MTB-R is positions 192–210 of the V2 region located within the 16 S rRNA gene [20]. Amplification was achieved using 1·7 mM $MgCl_2$, 200 μM dNTPs, and 0·4 μM of each primer in a total volume of 50 μl. The amplification procedure consisted of 36 cycles at 94 °C/1 min, 58 °C/1 min and 72 °C/2 min followed by a final extension step at 72 °C/5 min.

Multiplex-PCR

Two gene fragments were amplified using a multiplex-PCR. One of these amplified with primer set RAC1/RAC8, the other coding for 1031 bp of the RD4 *M. tuberculosis*; this region was synthesized using the primer combination previously reported (Y277-32F5′-GACATGTACGAGAGACGGCATGAG-3′/Y277-32R5′-AATCCAACACGCAGCAACCAG-3′) [15]. Multiplex-PCR reactions were carried out using 1·5 mM $MgCl_2$, 200 μM dNTPs, 10% DMSO, 0·1 μM of primers RAC1/RAC8 and 0·4 μM of primers Y277-32F/Y277-32R. The amplification procedure consisted of 36 cycles at 95 °C/30 s, 55 °C/1 min and 72 °C/2 min followed by a final extension step at 72 °C/5 min. All PCR tests were repeated at least three times.

Amplification products were run in a 1% neutral agarose gel and TBE (89 mM Tris, 89 mM borate, 2 mM EDTA). In all cases, a 1 kb plus DNA ladder (Life Technologies, Rockville, MD, USA) was used as molecular-size marker. Attempts to find conditions

https://doi.org/10.1017/S095026880300829X Published online by Cambridge University Press

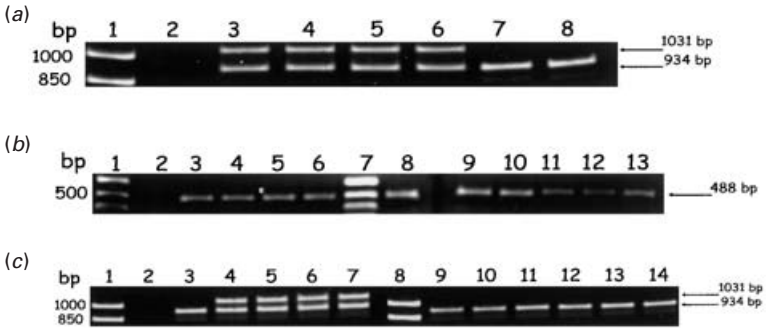

Fig. 2. Amplification by PCR of genomic regions of *M. tuberculosis* complex members and some *M. bovis* and *M. tuberculosis* clinical isolates. (*a*) Products obtained by amplifying two fragments (multiplex-PCR); one corresponding to 1031 bp of the RD4 region (primers Y27732F/Y25532R) [15], and another from the last 99 codons of the *murA* gene to position 357 of the 16 S rRNA gene (primers RAC1/RAC8. Lane 1, molecular-size markers; lane 2, no DNA; lane 3, *M. africanum*; lane 4, *M. canetti*; lane 5, *M. microti*; lane 6, *M. tuberculosis*; lane 7, *M. bovis*; lane 8, *M. bovis* BCG. (*b*) Products obtained by amplifying the region from the last five codons of *murA* gene, to the V2 region of the 16 S rRNA gene (primers MTB-F/MTB-R). Lane 1, molecular-size markers; lane 2, no DNA; lane 3, *M. bovis* AN5; lanes 4–6, *M. tuberculosis* clinical isolates; lane 7, molecular-size markers; lanes 8–10, *M. bovis* human clinical isolates; lanes 11–13, *M. bovis* bovine clinical isolates. (*c*) Products obtained by amplifying two fragments (multiplex-PCR); one corresponding to 1031 bp of the RD4 region (primers Y27732F/Y25532R) [15], and another from the last 99 codons of the *murA* gene to position 357 of the 16 S rRNA gene (primers RAC1/RAC8). Lane 1, molecular-size markers; lane 2, no DNA; lanes 3, *M. bovis* AN5; lane 4, *M. tuberculosis* H37Rv; lanes 5–7, *M. tuberculosis* clinical isolates; lane 8, molecular-size markers; lanes 9–11, *M. bovis* human clinical isolates; lanes 12–14, *M. bovis* bovine clinical isolates.

where all three set of primers (RAC1/RAC8, MTB-F/MTB-R and Y277-32F/Y277-32R) could be used in a single reaction were not successful.

RESULTS

One of the regions of the genome of interest (the *murA*/16 S rRNA intergenic region) and the primers used for amplification by PCR are illustrated in Figure 1*a*.

Figure 1*b* shows the amplification products of all bacterial strains using primers RAC1/RAC8. Only those bacteria belonging to the genus *Mycobacterium* (*M. africanum*, *M. bovis*, *M. bovis* BCG, *M. canetii*, *M. microti*, *M. tuberculosis* H37Rv, *M. avium*, *M. leprae*, *M. fortuitum*, *M. phlei* and *M. smegmatis*) showed an amplification product, the size depending on the species under investigation [17] (Fig. 1*b*, lanes 3–8 and 10–14). Members of the *M. tuberculosis* complex yielded a product of the expected size (934 bp) (Fig. 1*b*, lanes 3–8). No product was observed when DNA from *B. megaterium*, *B. subtilis* and *S. coelicolor* was amplified using RAC1/RAC8 (data not shown), confirming the specificity of primers RAC1/RAC8.

Figure 1*c* shows that the MTB-F/MTB-R primers amplified a region of 488 bp only in members of the *M. tuberculosis* complex (lanes 3–8). No PCR product was obtained when the substrate was genomic DNA from another mycobacteria: *M. avium*, *M. leprae*, *M. fortuitum*, *M. phlei* or *M. smegmatis* (lanes 10–14). It has been demonstrated by sequencing studies that the region amplified by this set of primers is identical in all members of MTC (unpublished observations). Likewise, no PCR product was obtained when samples from other genera were analysed, namely, *B. megaterium*, *B. subtilis* or *S. coelicolor* (data not shown). These results strongly suggest that the MTB-F/MTB-R set of primers is specific for the five members of the MTC.

The results of multiplex-PCR (with primers RAC1/RAC8 and Y277-32F/Y277-32R) are shown in Figure 2*a*. As expected two fragments were amplified when genomic DNA from *M. africanum*, *M. canetti*, *M. microti* and *M. tuberculosis* were used: one of 934 bp (RAC1/RAC8) and another of 1031 bp (RD4) (lanes 3–6), whereas from genomic DNA of *M. bovis* AN5 and *M. bovis* BCG only a single 934 bp fragment was amplified (lanes 7 and 8). These results showed that the multiplex-PCR (RAC1/RAC8 and Y277-32F/Y277-32R) can be used to distinguish between *M. bovis* and other members of the MTC, and when combined with the single PCR using MTB-F/MTB-R primers, the identification of *M. bovis* is indeed unambiguous.

When primers MTB-F/MTB-R were used, all 58 bovine and 23 human clinical isolates amplified the expected product (488 bp). Figure 2*b* shows three

https://doi.org/10.1017/S095026880300829X Published online by Cambridge University Press

M. tuberculosis human isolates (lanes 4–6), three *M. bovis* human isolates (lanes 8–10) and three *M. bovis* bovine isolates (lanes 11–13).

When the multiplex PCR was applied to DNA from isolates mentioned in Figure 2*b*, all *M. bovis* isolates regardless of origin yielded a single product of 934 bp, corresponding to the size expected using primers RAC1/RAC8; Figure 2*c* (lanes 9–14), shows the results from some of these clinical isolates. Two products were obtained: one of 934 bp and other of 1031 bp (a product of RD4) when genomic DNA from the 20 *M. tuberculosis* isolates was analysed. Figure 2*c* (lanes 5–7) shows the results obtained with three *M. tuberculosis* clinical isolates.

DISCUSSION

In this work we have developed a useful and practical strategy for the identification of clinical isolates of *M. bovis* and its differentiation from other members of the MTC. The method is based on the amplification of DNA sequences by PCR using primers of proven reliability.

We propose that this technique could be used both as an alternative tool for the identification of *M. bovis* in veterinary and human clinical isolates and as a feasible test for epidemiological studies in Latin American countries such as Mexico, where a lack of reliable data about the prevalence of *M. bovis* in human tuberculosis is notorious.

Our procedure does not distinguish between *M. bovis* and *M. bovis* BCG. In most cases *M. bovis* BCG is not found in clinical specimens; however, in immunosuppressed patients, *M. bovis* BCG can be pathogenic. In these cases an additional amplification exploring the presence or absence of the region RD1 [22] can be carried out to identify *M. bovis* BCG.

The clinical samples of *M. bovis* were not tested for their resistance towards pyrazinamide because there is no indication that any resistance or sensitivity affects the nucleotide sequences of the regions of DNA that were selected for amplification. However, the 58 bovine and 3 human *M. bovis* clinical isolates are now being examined for their resistance to pyrazinamide.

Other procedures based on PCR amplification of some genomic regions such as IS1081 [23], MPB70 [24], MPT40 [25] or a 500 bp region [26–28], have been proposed to identify *M. bovis*. However, all these techniques are not entirely specific for *M. bovis*, since the regions amplified are not present exclusively in *M. bovis*. For instance, MPT40 is present in most, but not all, *M. tuberculosis* isolates [29, 30], and can be present in some *M. bovis* isolates [30].

Recently, two new methods that can distinguish between *M. bovis* and *M. tuberculosis* have been developed: One is based on a multiplex-PCR that amplifies a region of the *pncA* gene that is mutated in *M. bovis* [31]; the other is based on a PCR amplification of some regions of difference (RD) [32]. Our test can give more information, since it can identify whether or not a pathogen is a member of the *M. tuberculosis* complex, and since it is also specific for *M. bovis*.

ACKNOWLEDGEMENTS

This work was supported by the European Commission Science Research and Development Programme, contract number CT-2002-100063; CONACyT, grants 37814-N and 31612-N; and CEGEPI, IPN, 2002708 and 32.04. L.C-M. received financial support from CONACyT. I.E-G and J.A. G-y-M are fellows of COFAA, EDI and SNI. We thank Dr Robert A. Cox and Dr Leopoldo Flores for critically reviewing the manuscript, and Dr M. J. Colston and Dr C. Hernandez, for gifts of the DNA samples.

REFERENCES

1. Grange JM, Yates MD. Zoonotic aspects of *Mycobacterium bovis* infection. Vet Microb 1994; **40**: 137–51.
2. De Kantor I, Ritacco V. Bovine tuberculosis in Latin America and the Caribbean: current status, control and eradication programs. Vet Microb 1994; **40**: 5–14.
3. Hancoux M. Bovine tuberculosis: milk and meat safety. Lancet 2002; **359**: 706–7.
4. Cosivi O, Grange JM, Daborn CJ, et al. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg Infect Dis 1998; **4**: 59–70.
5. Milian F, Sanchez LM, Toledo P, Ramirez C, Santillan MA. Descriptive study of human and bovine tuberculosis in Queretaro, Mexico. Rev Latin-American Microbiol 2000; **42**: 13–9.
6. Van Deutekom H, Smulders YM, Roozendaal KJ, van Soolingen D. Bacille Calmette Guérin (BCG) meningitis in AIDS patient 12 years after vaccination with BCG. Clin Infect Dis 1996; **22**: 870–1.
7. Grifith DE. Mycobacteria as a pathogens of respiratory infection. Infect Dis Clin North Am 1998; **12**: 593–622.
8. Brosch R, Gordon SV, Marimesse M, et al. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci 2002; **99**: 3684–9.
9. Corner LA. Postmortem diagnosis of *Mycobacterium bovis* infection in cattle. Vet Microb 1994; **40**: 53–63.
10. Collins DM, Erasmuson SK, Stephens DM, Yates GF, Lisle GW. DNA fingerprinting of *Mycobacterium bovis*

strains by restriction fragment analysis and hybridization with insertion elements IS1081 and IS6110. J Clin Microbiol 1993; **31**: 1143–7.
11. Da Silva A, da Costa L, Torres HM, et al. Use of PCR-restriction fragment length polymorphism analysis of the hsp65 gene for rapid identification of mycobacteria in Brazil. J Microbiol Meth 1999; **37**: 223–9.
12. Niemann S, Harmsen D, Rüsch-Gerdes S, Richter E. Differentiation of clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis. J Clin Microbiol 2000; **38**: 3231–4.
13. Telenti A, Marches F, Balz M, Bally F, Böttger E, Bodmer T. Rapid identification of Mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993; **31**: 175–8.
14. Kamerbeek J, Scholus L, Kolk A, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997; **35**: 907–14.
15. Gordon S, Brosch R, Billault A, Garnier T, Eiglmeier K, Cole ST. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol Microbiol 1999; **32**: 643–55.
16. González-y-Merchand JA, Colston MJ, Cox RA. The rRNA operon of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*: comparison of promoter elements and of neighbouring upstream genes. Microbiology 1996; **142**: 667–74.
17. Gonzalez y Merchand JA, García MJ, Gonzalez S, Colston MJ, Cox RA. Strategies used by pathogenic and non-pathogenic mycobacteria to synthesize rRNA. J Bacteriol 1997; **179**: 6949–58.
18. Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999; **284**: 1520–3.
19. Kempsell E, Ji YE, Estrada IC, Colston MJ, Cox RA. The nucleotide sequence of the promoter, 16S rRNA and spacer region of the ribosomal RNA operon of *Mycobacterium tuberculosis* and comparison with *Mycobacterium leprae* precursor rRNA. J Gen Microbiol 1992; **138**: 1717–22.
20. Kirschner P, Springer B, Vogel U, et al. Genotypic identification of Mycobacteria by nucleic acid sequence determination: Report of a 2-year experience in a clinical laboratory. J Clin Microbiol 1993; **31**: 2882–9.
21. Stonebrink B. Tubercle bacilli and pyruvic acid. Proc Tuberc Res Coun 1957; **44**: 67.
22. Talbot EA, Williams DA, Frothigham R. PCR identification of *Mycobacterium bovis* BCG. J Clin Microbiol 1997; **35**: 566–9.
23. Cousins DV, Wilton SD, Francis BR. Use of DNA amplification for the rapid identification of *Mycobacterium bovis*. Vet Microbiol 1991; **27**: 187–95.
24. Wards BJ, Collins DM, de Lisle GW. Detection of *Mycobacterium bovis* in tissues by polymerase chain reaction. Vet Microbiol 1995; **43**: 227–40.
25. Del Portillo P, Thomas MC, Martínez E, et al. Multiprimer PCR system for differential identification of Mycobacteria in clinical samples. J Clin Microbiol 1996; **34**: 324–8.
26. Rodríguez JG, Mejia GA, Del Portillo P, Patarroyo ME, Murillo LA. Species-specific identification of *Mycobacterium bovis* by PCR. Microbiology 1995; **141**: 2131–8.
27. Rodriguez JG, Fissanoti JC, Del Portillo P, Patarroyo ME, Romano MI, Cataldi A. Amplification of a 500-base-pair fragment from cultured isolates of *Mycobacterium bovis*. J Clin Microbiol 1999; **37**: 2330–2.
28. Romero RE, Garzon DL, Mejía GA, Monroy W, Patarroyo ME, Murillo LA. Identification of *Mycobacterium bovis* in bovine clinical samples by PCR species-specific primers. Can J Vet Res 1999; **63**: 101–6.
29. Liébana E, Aranaz A, Francis B, Cousins D. Assessment of genetic markers for species differentiation within the *Mycobacterium tuberculosis* complex. J Clin Microbiol 1996; **34**: 933–8.
30. Weil A, Plikaytis BB, Butler WR, Woodley CL, Shinick TM. The mpt40 gene is not present in all strains of *Mycobacterium tuberculosis*. J Clin Microbiol 1996; **34**: 2309–11.
31. Shah DH, Verma R, Bakshi C, Singh RK. A multiplex-PCR for the identification of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. FEMS Microbiol Lett 2002; **214**: 39–43.
32. Parsons LM, Brosch R, Cole ST, et al. Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. J Clin Microbiol 2002; **40**: 2339–45.