

BCG Infection due to MPT64-Negative Strain: A Diagnostic Challenge

Parasmal Suresh,¹ Lalitha Biswas,¹ Vinitha Prasad,² Anil Kumar,^{3*} Suchitra Sivadas,² Sadia Khan,³ and Raja Biswas¹

¹Centre for Nanosciences and Molecular Medicine, Amrita Institute of Medical Sciences and Research Centre, Amrita VishwaVidyapeetham, Kochi, India; ²Department of Paediatrics, Amrita Institute of Medical Sciences and Research Centre, Kochi, India; ³Department of Microbiology, Amrita Institute of Medical Sciences and Research Centre, Kochi, India

Abstract. MPT64 is a 24-kDa immunogenic protein that is widely used as a diagnostic marker for the differentiation of *Mycobacterium tuberculosis* complex (MTBC) from nontuberculous *Mycobacterium* (NTM). Unlike *Mycobacterium bovis*, Bacillus Calmette-Guerin (BCG) vaccine strains with RD2 deletion do not secrete MPT64. Culture isolates from infections due to these strains may be falsely identified as nontuberculous *Mycobacterium* in the absence of clinical correlation. Here, we present one case each of BCG adenitis and osteitis, both of which were considered as MPT64 card-negative *Mycobacterium* spp. (i.e., NTM) and were later identified as *M. bovis* BCG Danish 1331 strain. The first case was a 4-month-old female infant admitted with swollen lymph nodes in the left supraclavicular and the left axillary region of 1 month duration. The second case was of a 1-year-and-5-month-old male child who presented with a limp on the left leg and soft tissue swelling of 1 month duration on the anterolateral aspect of the left knee joint. In both cases, BCG vaccine was administered at birth on the left deltoid region and had healed without any complication. Clinical samples in both cases were positive by Xpert tuberculosis/RIF for MTBC, and cultures grew acid-fast bacilli which were negative by MPT64 assay. The clinical implication of infections due to *M. bovis* BCG is immense as they are inherently resistant to pyrazinamide, and the presence of disseminated BCG infection in young children is a hallmark of serious immune deficiency which needs to be ruled out.

INTRODUCTION

Live attenuated BCG vaccine was introduced to the world in 1921.¹ In countries like India, with a high burden of tuberculosis (TB), the WHO recommends BCG vaccination of all children at birth to prevent tuberculous meningitis and disseminated TB in young children and infants.¹ Among the various BCG strains, Glaxo 1077, Pasteur 1173, Danish 1331, and Tokyo 172 account for almost 90% of the vaccines worldwide.^{1,2} Whereas Pasteur 1173 and Danish 1331 are considered as strong strains (higher vaccine efficacy), Glaxo 1077 and Tokyo 172 are weak strains (lower vaccine efficacy).^{1,2} The genealogy of BCG vaccine strains based on historical data shows that BCG strains differ in the presence of *mpb64* gene and the number of copies of *IS6110* gene.^{3,4} The ancestral BCG strain obtained before 1925 had two copies of *IS6110* and *mpb64* genes, but subsequently loss of one *IS6110* happened in 1925–1926 followed by loss of *mpb64* gene around 1927–1931.^{3,4} The *mpb64* gene is present in BCG-Japan, BCG-Sweden, BCG-Moreau, BCG-Birkhaug, and BCG-Russia, whereas it is absent in BCG-Danish, BCG-Pasteur, BCG-Glaxo, BCG-Tice, and BCG-Connaught. Complications due to BCG vaccination are influenced by BCG strain, dose, age, technique of immunization, and the underlying immune status of the vaccines.^{2,3} The incidence of severe complications ranges from 2 to 1,000 per million vaccinations and is most often seen in patients with primary immunodeficiency disorders such as chronic granulomatous disease, severe combined immunodeficiency, type 1 cytokine axis defect, DiGeorge syndrome, and HIV infection.^{1,3} Severe complications may require chemotherapy with anti-TB drugs, which get complicated by the inherent resistance to pyrazinamide in all *Mycobacterium bovis* strains.^{1,5} Because of inherent resistance to pyrazinamide, the CDC recommends a triple-drug regimen consisting of rifampicin, isoniazid, and

ethambutol with treatment duration being extended to 9 months for treatment of disease due to *M. bovis*.⁶ Disseminated BCG infection in young children could be an indication of serious immune deficiency, which needs to be investigated and ruled out. Therefore, differentiation of *M. bovis* BCG from *Mycobacterium tuberculosis* as well as from nontuberculous mycobacteria (NTM) is crucial for appropriate treatment. Assays for MPT64 protein have been recommended by the Foundation for Innovative New Diagnostics for rapid identification of the *M. tuberculosis* complex using the BACTEC MGIT 960 liquid culture system (Becton Dickinson Diagnostics, Sparks, MD).⁷ The assays are routinely performed all over the world, both in developed and developing countries, as suggested by two meta-analyses on their performance in accurately identifying the *M. tuberculosis* complex in liquid culture.^{8,9} We report two cases of BCG infection due to *M. bovis* BCG strains which were MPT64 negative.

SHORT REPORT

Case 1. A 4-month-old female infant was admitted with complaints of high-grade intermittent fever, cough, and difficulty breathing. A swelling in the neck and the axillary region was noticed 3 months earlier. She was also diagnosed to have asplenia and patent ductus arteriosus at the age of 3 months. Clinical examination revealed bilateral inguinal hernia, coarse facies, and matted lymph nodes in the left axilla and the supraclavicular region. BCG vaccine administered on day 2 of life on the left deltoid region had healed without any complications. Chest X-ray showed clear lung fields without any hilar nodes or mediastinal widening.

Case 2. A 1-year-and-5-month-old male child, developmentally normal for age, presented with a limp on the left leg and soft tissue swelling of 1 month duration on the anterolateral aspect of the left knee joint. The size of the swelling progressively increased over 2 weeks from about 1 × 1 cm to 2 × 2 cm. There were no complaints of associated fever, pain over swelling, skin changes, or weight loss. The child was immunocompetent with no underlying disorders, and the BCG

* Address correspondence to Anil Kumar, Department of Microbiology, Amrita Institute of Medical Sciences and Research Centre, Amrita VishwaVidyapeetham, Ponekkara, Kochi 682041, India. E-mail: vanilkumar@aims.amrita.edu

vaccine administered on the left deltoid region at birth healed without any complication.

Written informed consent was obtained from the legal guardians of both the patients. Pus aspirated from the left supraclavicular lymph node in the first case and tissue from the excised swelling in the second case were subjected to acid-fast stain, aerobic cultures, *Mycobacterium tuberculosis* complex (MTBC) PCR using Xpert[®] MTB/RIF assay (Cepheid Inc., Sunnyvale, CA), and liquid mycobacterial cultures using BACTEC MGIT 960 (Becton Dickinson Diagnostics, Sparks, MD). The clinical mycobacterial isolates were compared with *M. tuberculosis* H37Ra, MPT64-positive *M. bovis* BCG-Russia (Moscow 368, Serum Institute of India), and MPT64-negative *M. bovis* BCG-Denmark (Danish 1331, Green Signal Bio Pharma Pvt Ltd. Chennai, India) strains because in India both these strains are used for vaccination. Genomic DNA was isolated from the cultures of clinical isolates, vaccine strains, and *M. tuberculosis* H37Ra. To differentiate between *M. bovis* and the other MTBC complex organisms, *gyrB* gene was amplified by PCR and the 1,020-bp PCR product was subjected to restriction fragment length polymorphism (RFLP) using *TaqI* and sequencing. The sequences obtained were analyzed using the BLASTN algorithm. To detect and differentiate *M. bovis* BCG from *M. tuberculosis* and *M. bovis*, PCR amplifications of the RD1 and RD4 regions were performed using the primers, as described earlier (Table 1).¹⁰

Smear for acid-fast bacilli (AFB) and aerobic cultures of the pus and tissue were negative. Mycobacterial cultures grew AFB showing cord formation. PCR of pus and tissue using Xpert MTB/RIF was positive for MTBC with rifampicin resistance not detected, indicating that both the specimens belong to MTBC. But both the MGIT culture isolates were negative for the MPT64 protein immunochromatographic test (ICT) (Standard Diagnostics, Seoul, South Korea), suggestive of NTM. To resolve these conflicting results and identify the species of these two MPT64 card-negative and Xpert MTB/RIF-positive MGIT culture isolates, PCR amplification, RFLP, and sequencing methods were carried out.

RFLP patterns of the clinical isolates were identical to those of the vaccine strains using *TaqI* (Figure 1A and B). The RD1 PCR was used to differentiate *M. bovis* BCG (196 bp) from *M. tuberculosis*/*M. bovis* (146 bp). The RD4 PCR was used to differentiate between *M. tuberculosis* (172 bp) and *M. bovis*/*M. bovis* BCG (268 bp) (Figure 1E and F). RD4 PCR results identified the culture isolates as *M. bovis*/*M. bovis* BCG (268 bp), and RD1 PCR identified them as *M. bovis* BCG. Sequencing of the *gyrB* and the RD4 PCR products identified the clinical isolates as *M. bovis* BCG Danish 1331 (Figure 1B and C).

Based on these observations, the first patient underwent aspiration of the supraclavicular lymph node and biopsy of the

left axillary lymph node, whereas the second patient underwent excision of the soft tissue swelling and treatment with modified first-line antitubercular drugs with replacement of pyrazinamide with streptomycin. At 1-year follow-up, the first child is doing well with complete resolution of lymphadenitis without any relapse. The second child completed 6 months of antitubercular therapy with complete resolution of symptoms.

DISCUSSION

The identification of *M. bovis* BCG is not straightforward as it belongs to MTBC, a group of highly related organisms comprising *M. tuberculosis*, *Mycobacterium africanum*, *M. bovis*, *Mycobacterium microti*, and *Mycobacterium canettii*.¹¹ The viable and actively dividing cells of the members of MTBC secrete a 24-kDa secretory protein which remains stable in the culture medium for at least 1 year.^{2,7} The introduction of rapid ICT for the detection of MPT64 protein has made the differentiation of MTBC from NTM very simple and economical.⁸ However, *M. bovis* BCG strains with deletion or mutation in the *mpb64* gene cannot be detected with this assay.⁴ Single-nucleotide polymorphisms that exist in the *gyrB* gene of the MTBC were used to differentiate *M. bovis* from the other MTBC species by performing RFLP using the restriction enzyme *TaqI*.¹² Previous studies have shown the absence of regions of difference (RD) RD1, RD9, and RD10 in all BCG vaccine strains and the presence in TB strains, although *M. bovis* lacks the RD4 region. Non-BCG *M. bovis* is distinguished from BCG by the presence of RD1 and the absence of RD9.¹⁰ In this study, we have used the RD1 region- and RD4 region-specific amplifications to differentiate between *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG.

The diagnostic algorithm for pediatric TB in India advocates the upfront use of cartridge-based nucleic acid testing (Xpert MTB/RIF) and cultures, if available.¹³ Both these tests will not differentiate *M. tuberculosis* from *M. bovis* and may lead to inappropriate treatment with pyrazinamide, to which *M. bovis* is inherently resistant. Moreover, the use of MPT64-negative BCG strain will lead to misidentification as NTM in mycobacterial cultures. Pediatric infections due to NTM are very rare and may occur in children with underlying immunodeficiency. A study from India reported that 75% of infants with BCG-related disease have regional or localized involvement, whereas only 25% had disseminated disease.¹⁴ Among patients with disseminated disease, only one patient had *IFN γ -R1* gene mutation.¹⁴ The use of two different strains for vaccination in the same population, one with and the other without the *mpb64* gene, further complicates the problem of identification. In India, the public sector uses MPT64-negative

TABLE 1
List of tests performed to examine the specimens and culture isolates

Method	Test performed	Result
Microbiological identification (clinical specimen)	Acid-fast staining	Negative
	BACTEC MGIT liquid mycobacterial culturing	Positive
Rapid Methods (PCR/immunochromatographic)	Xpert MTB/RIF (clinical specimen)	Positive for MTBC/RIF sensitive
	SD Biotline MPT64 card assay (culture isolates)	Negative for MTBC
Molecular biological identification (culture isolates)	<i>gyrB</i> PCR-RFLP using <i>TaqI</i>	<i>M. bovis</i>
	RD1	<i>M. bovis</i> BCG
	RD4	<i>M. bovis</i> / <i>M. bovis</i> BCG
	DNA sequencing <i>gyrB</i> gene	<i>M. bovis</i> BCG Danish 1331

M. bovis = *Mycobacterium bovis*; MTBC = *Mycobacterium tuberculosis* complex.

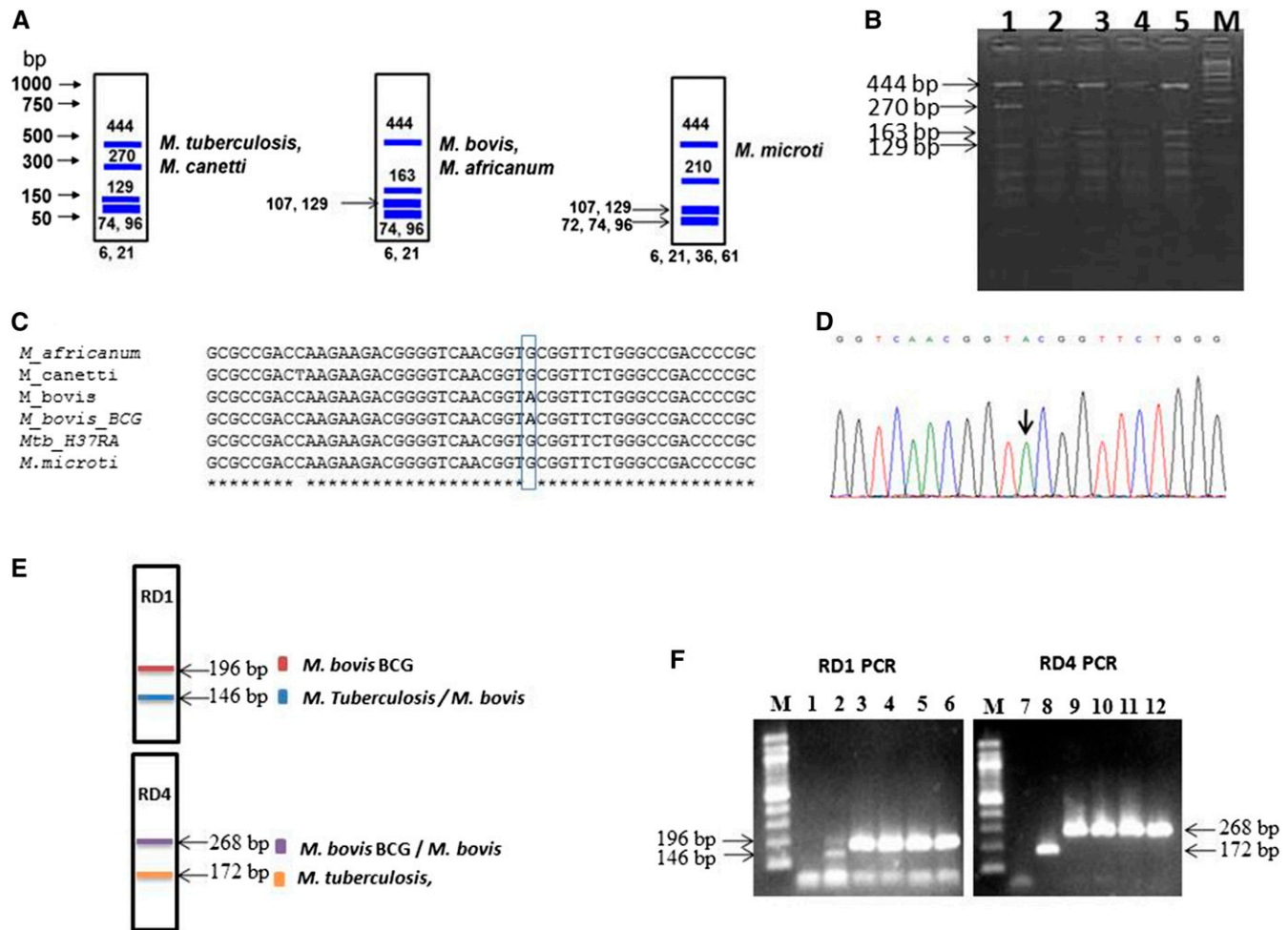


FIGURE 1. (A) Schematic representation of the *TaqI* restriction pattern of the 1,020-bp *gyrB* fragment among the members of the MTBC. (B) RFLP pattern obtained after the digestion of the 1,020-bp *gyrB* PCR product with the *TaqI* restriction enzyme. Lane 1: *Mycobacterium tuberculosis* H37Ra, 2: *Mycobacterium bovis* BCG-Russia (Moscow 368) (Serum Institute of India), 3: *M. bovis* BCG-Denmark (Danish 1331) (Green Signal Biopharma, India), 4 and 5: MGIT culture-positive and MPT64-negative samples, Lane M: 100-bp DNA ladder. A 1020-bp PCR-amplified product was seen in both clinical isolates. (C) Multiple sequence alignment of the discriminatory regions in the *gyrB* gene between *M. bovis* and *M. bovis* BCG and other members of the MTBC. The black box indicates the single nucleotide variation G to A. (D) Electropherogram of the *gyrB* PCR product of the clinical isolate. The arrow indicates the single-nucleotide variation G to A representing *M. bovis*. (E) Schematic representation of RD1 and RD4 PCR amplification band sizes for differentially identifying *M. bovis* BCG and *M. tuberculosis* and *M. bovis*. (F) Agarose gel electrophoresis of RD1 and RD4 PCR products: Lanes 1 to 6 are RD1 PCR products; Lane M: 100-bp DNA ladder, Lane 1: no template control (NTC), Lane 2: *M. tuberculosis* H37Ra, Lane 3: *M. bovis* BCG-Russia (Moscow 368), Lane 4: *M. bovis* BCG-Denmark (Danish 1331), and Lanes 5 and 6: MGIT culture-positive and MPT64-negative samples; Lanes 7 to 12 are RD4 PCR products; Lane 7: NTC, Lane 8: *M. tuberculosis* H37Ra, Lane 9: *M. bovis* BCG-Russia (Moscow 368), and Lane 10: *M. bovis* BCG-Denmark (Danish 1331); and Lanes 11 and 12: MGIT culture-positive and MPT64-negative samples. This figure appears in color at www.ajtmh.org.

Danish 1331 strain which is provided free of cost, whereas the private sector uses the MPT64-positive Russian (Moscow) 368 strain and needs to be bought by the patients.¹⁵ The knowledge of various strains used for vaccination plays a crucial role in avoiding misidentification in cases of BCG-associated complications. In our case, although the patient was asplenic, she was immunocompetent, and the BCG vaccine lesion healed normally with a scar. The children were vaccinated with Danish 1331 strain; therefore, the culture isolates were negative for the MPT64 antigen. The pus and tissue samples were positive for MTBC using Xpert MTB/RIF as it targets the *rhoB* gene for identification. However, Xpert MTB/RIF does not differentiate between the members of MTBC, making it not possible to identify *M. bovis* BCG. Because *M. bovis* is inherently resistant to pyrazinamide, accurate identification is necessary for appropriate chemotherapy.

Our case emphasizes the importance of careful use of the MPT64 kit with clinical correlation and combining morphological characteristics or nucleic acid amplification tests to identify MPT64-negative *M. bovis* BCG clinical isolates. Clinicians should also be aware that finding disseminated BCG infection in young children is a hallmark of serious immune deficiency which would need investigation and management.

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Authors' addresses: Parasamal Suresh, Raja Biswas and Lalitha Biswas, Center for Nanosciences and Molecular Medicine, Amrita

Institute of Medical Sciences and Research Centre, Amrita Vishwa Vidyapeetham, Kochi, India, E-mails: parasmals@aims.amrita.edu, rajabiswas@aims.amrita.edu and biswaslalitha@gmail.com. Vinitha Prasad and Suchitra Sivasdas, Department of Paediatrics, Amrita Institute of Medical Sciences and Research Centre, Amrita Vishwa Vidyapeetham, Kochi, India, E-mails: vinithaprasad@aims.amrita.edu and suchitra@aims.amrita.edu. Anil kumar, and Sadia Khan, Department of Microbiology, Amrita Institute of Medical Sciences and Research Centre, Kochi, India, E-mails: vanilkumar@aims.amrita.edu, and sadiakhan20004@aims.amrita.edu.

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