Frequency of Bacille Calmette-Guérin (BCG) and Mycobacterium tuberculosis in Tissue Biopsy Specimens of Children Vaccinated With BCG

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Key Words: Bacille Calmette-Guérin; BCG; Mycobacterium tuberculosis; Disseminated mycobacterial infection; Lymph node; Real-time polymerase chain reaction; Melting curve analysis

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Abstract

Vaccination of all newborns with bacille Calmette-Guérin (BCG) vaccine is a standard practice in developing countries. Disseminated mycobacterial infection in an immunocompromised child can be caused by BCG and other mycobacteria.

A total of 21 patients with a histopathologic diagnosis of mycobacterial infection were studied in a period of 4 years. DNA was extracted from formalin-fixed, paraffin-embedded tissues. Real-time polymerase chain reaction was performed to determine the mycobacterial species. The overall sensitivity of the assay was 71.5%. The prevalence rates of BCG, Mycobacterium tuberculosis, and other mycobacteria in the positive results were 80% (12/15), 13% (2/15), and 7% (1/15), respectively.

Bacille Calmette-Guérin (BCG) is an attenuated strain of Mycobacterium bovis introduced as a human vaccine against tuberculosis in the early 1920s.1,2 Vaccination of all newborns with BCG vaccine is a standard practice in Iran and other developing countries, and all newborns receive the vaccine in the first or second day of life. This practice is generally considered safe, but rare complications may occur in approximately 1.9% of cases.3 These complications include regional lymphadenopathy, subcutaneous abscess, osteomyelitis, eczema vaccinatum, hypertrophic scars, and keloid formation, which are often self-limiting, and usually no treatment is necessary.3,4 Most lymphadenopathies related to BCG complications are subclinical and regress spontaneously in immunocompetent patients.3 On the other hand, severe disseminated BCG infection may occur in children with defective immunity and HIV infection.5,6 The incidence of disseminated BCG infection has been reported between 0.01 and 3.4 per million in different studies.1,7,8 Disseminated mycobacterial infection in an immunocompromised child can also be caused by mycobacteria other than BCG.9-11 Mycobacterium tuberculosis infects about one third of the world’s population each year and is prevalent in areas in which BCG vaccination is recommended.7,12 Meanwhile nontuberculous mycobacteria are widespread in the environment and a cause of opportunistic infection in immunocompromised hosts.13

In addition to rapidity, molecular techniques are confirmatory for the presence of the exact type of mycobacterial infection. The ability to rapidly and specifically identify BCG is clinically important because of different treatment

Upon completion of this activity you will be able to:

• describe the predisposing factors of disseminated bacille Calmette-Guérin (BCG) infection and why it is important to differentiate BCG infection from Mycobacterium tuberculosis infection.
• list the advantages of molecular assays and strategies for increasing assay sensitivity for detection of mycobacteria in tissue specimens.
• apply polymerase chain reaction data to determine the mycobacterial species.

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schemes. On the other hand, in many situations, the physician is unaware of the infectious nature of the disease before the result of the microscopic examination of the tissue is reported; therefore, often no tissue is sent for culture at the time of biopsy. This is an important problem, especially in cases in which mycobacterial infection is found in autopsy material; therefore, establishing a method for the diagnosis of the specific type of mycobacterial infection in formalin-fixed, paraffin-embedded tissue seems mandatory.

To our knowledge, there no published studies about the frequency of BCG and other mycobacterial infections in Iranian children. The aim of this study was to determine the frequency of BCG and other mycobacteria in biopsy materials in which the Ziehl-Neelsen stain was positive for acid-fast bacilli.

**Materials and Methods**

The cases were collected in a period of 4 years, 2004 to 2008, in a children’s medical center hospital. In addition to being a referral tertiary care center, this hospital is the major teaching center of Tehran University of Medical Sciences, Tehran, Iran. Patients are admitted from all regions of Iran, representing a wide spectrum of socioeconomic levels.

A total of 23 patients with a histopathologic diagnosis of mycobacterial infection were identified. Patients’ clinical records were studied. Of 23 selected cases, 2 had limited tissue and were excluded from the study. Paraffin blocks of 21 remaining cases were suitable for the study and were retrieved. The H&E- and Ziehl-Neelsen–stained slides were reviewed. DNA was extracted from formalin-fixed, paraffin-embedded tissues using a previously described method.

Two rounds of real-time polymerase chain reaction (PCR) were performed. The flowchart for identification is shown in Figure 1. The first round of real-time PCR was performed using *Mycobacterium* genus control primers as described by Pinsky and Banaei. The procedures of block sectioning, extraction, and PCR were repeated for the negative samples. This was done owing to uneven distribution of acid-fast bacilli in the specimen. The positive samples were subjected to a second round of real-time PCR using *Mycobacterium tuberculosis* and *M. bovis* BCG-specific primers as described by Pinsky and Banaei.

**Extraction**

Three consecutive 5-μm sections were made using disposable blades, and then the paraffin sections were transferred to sterile 1.5-mL Eppendorff tubes by using a plastic applicator; a new blade and applicator were used for each block. Sections were pelleted by centrifugation of the tubes at 16,000 g for 1 minute, and then 200 μL of sterile distilled water with 0.5% vol/vol polysorbate 20 was added. The tubes were placed in a heating block at 100°C for 10 minutes and then snap frozen in liquid nitrogen. The cycle of boiling and freezing was repeated 2 times, and the tubes were boiled again for 10 minutes and finally centrifuged at 3,000 g for 20 minutes. The supernatant was transferred to a fresh tube and stored at −20°C.

**Polymerase Chain Reaction:**

Each reaction volume was 20 μL and contained AccuPower Greenstar PreMix (Bioneer, Alameda, CA), 10 pmol of each primer, and 5 μL of template. Reaction 1 had primers to detect a region of the 16S ribosomal RNA gene that is common to all mycobacteria (*Mycobacterium* genus control primers). Reaction 2 had primers to detect the presence of RD9 (specific for *M. tuberculosis*) or the absence of RD1 (specific *M. bovis* BCG-specific primers; Table 1). Negative control, no template control, *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG control samples were included in each PCR run. The reactions were performed in a Rotor-Gene 3000...
Monajemzadeh et al / BCG and M. TUBERCULOSIS AFTER BCG VACCINATION

**Table 1**

Primer and Product Characteristics Used in This Study

<table>
<thead>
<tr>
<th>Product</th>
<th>Length (bp)</th>
<th>Mycobacterium</th>
<th>Melting Peak (°C)</th>
<th>Reaction</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD9 present</td>
<td>51</td>
<td>Tuberculosis</td>
<td>81</td>
<td>2</td>
<td>5-TTTCGAGCCGTAATTACTGTG; 5-GAGCATTTCTCGCTCCGAAAT; 5-GATTGTAGCCTGCTGGTCTTCT; 5-TCAACCGGTATCTCGGAAT</td>
</tr>
<tr>
<td>RD1 deleted</td>
<td>226</td>
<td>BCG</td>
<td>91.5</td>
<td>2</td>
<td>5-GGATTTGACGTCGTGCTTCT; 5-TTCAACCGGTATCTCGGAAT</td>
</tr>
<tr>
<td>Mycobacterial 16S rRNA</td>
<td>78</td>
<td>Genus control</td>
<td>84.5</td>
<td>1</td>
<td>5-CAACGCCGAAGAACCTTACCT; 5-TGACACACAGGCCAACAGGGA</td>
</tr>
</tbody>
</table>

BCG, bacille Calmette-Guérin; bp, base pairs; rRNA, ribosomal RNA.
* Designed by Pinsky and Banaci.17

real-time machine (Corbett Research, Mortlake, Australia) as follows: initial denaturation at 95°C for 5 minutes and 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Final extension involved 72°C for 5 minutes and a last step composed of a 60°C to 95°C temperature ramp at a rate of 1°C/second to generate the melting curve. Positive control experiments were run on 2% agarose gel to confirm the amplicon size.

**Results**

Of 21 selected patients, 10 were boys, and the rest were girls. Ages of patients at the time of admission ranged from 2 to 72 months with a mean and standard deviation of 20 and 25 months, respectively.

Two histomorphologic patterns were observed as follows: (1) well-circumscribed granulomas, with multinucleated giant cells and a scant number of acid-fast bacilli (type I reaction); and (2) ill-defined granulomas or diffuse histiocytic reaction with a large number of acid-fast bacilli (type II reaction).

These findings are consistent with previous studies.3 One of the patients (case 1) had bacteriologic confirmation of BCG infection before the present study. Of 21 samples, 6 were negative after 2 rounds of sectioning-extraction and PCR reaction 1. Twelve were positive for BCG, 2 were positive for tuberculosis, and the rest were negative in PCR reaction 2. The sensitivity of the assay was 71.5%. The prevalence rates of BCG, tuberculosis, and other mycobacteria in the positive samples were 80% (12/15), 13% (2/15), and 7% (1/15), respectively. The results are summarized in **Table 2**.

**Table 2**

Clinical Data and Results for First- and Second-Round Real-Time Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Case No./Sex/Age (mo)</th>
<th>Block No.</th>
<th>Result</th>
<th>Morphologic Reaction</th>
<th>Biopsy Site</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/6</td>
<td>83</td>
<td>2289</td>
<td>N</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>2/F/48</td>
<td>82</td>
<td>1650</td>
<td>N</td>
<td>Lymph node</td>
<td>NIMD</td>
</tr>
<tr>
<td>3/M/30</td>
<td>85</td>
<td>294</td>
<td>TB</td>
<td>Lung</td>
<td>NIMD</td>
</tr>
<tr>
<td>4/M/10</td>
<td>82</td>
<td>509A</td>
<td>BCG</td>
<td>Lymph node</td>
<td>NIMD</td>
</tr>
<tr>
<td>5/M/4</td>
<td>84</td>
<td>257</td>
<td>BCG</td>
<td>Lymph node</td>
<td>NIMD</td>
</tr>
<tr>
<td>6/F/7</td>
<td>84</td>
<td>1910</td>
<td>BCG</td>
<td>Appendix</td>
<td></td>
</tr>
<tr>
<td>7/F/14</td>
<td>83</td>
<td>374</td>
<td>BCG</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>8/M/6</td>
<td>83</td>
<td>1782</td>
<td>BCG</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>9/F/18</td>
<td>84</td>
<td>2294</td>
<td>BCG</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>10/F/72</td>
<td>82</td>
<td>385</td>
<td>TB</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>11/F/3</td>
<td>82</td>
<td>2082</td>
<td>BCG</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>12/M/5</td>
<td>82</td>
<td>1334</td>
<td>N</td>
<td>Liver</td>
<td>SCID*</td>
</tr>
<tr>
<td>13/M/15</td>
<td>86</td>
<td>1682</td>
<td>BCG</td>
<td>Paravertebral mass</td>
<td>SCID*</td>
</tr>
<tr>
<td>14/M/NA</td>
<td>85</td>
<td>5908</td>
<td>BCG</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>15/F/6</td>
<td>85</td>
<td>2306</td>
<td>M</td>
<td>Liver; spleen</td>
<td></td>
</tr>
<tr>
<td>16/F/5</td>
<td>84</td>
<td>512</td>
<td>N</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>17/M/4</td>
<td>85</td>
<td>1060</td>
<td>BCG</td>
<td>Liver</td>
<td>SCID*</td>
</tr>
<tr>
<td>18/F/8</td>
<td>86</td>
<td>591A</td>
<td>BCG</td>
<td>Liver; spleen</td>
<td></td>
</tr>
<tr>
<td>19/F/6</td>
<td>83</td>
<td>295</td>
<td>N</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>20/F/6</td>
<td>82</td>
<td>424</td>
<td>N</td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>21/M/5</td>
<td>86</td>
<td>590</td>
<td>N</td>
<td>Liver</td>
<td></td>
</tr>
</tbody>
</table>

BCG, bacille Calmette-Guérin; I, granulomatous; II, histiocytic; M, mycobacterium other than tuberculosis or BCG; N, negative; NA, not available; NIMD, no immunodeficiency found; SCID, severe combined immunodeficiency.
* Diagnosis based on clinical data, serum immunoglobulin level, and flow cytometry.
Discussion

BCG vaccination is contraindicated in infants with immunodeficiency; however, they are vaccinated before this diagnosis is made, and immunodeficiency may be diagnosed after the development of BCG complications.6 Meanwhile, proving this infection, even in autopsy materials, can improve the management of future siblings in affected families.

For Ziehl-Neelsen staining to become positive, biopsy material must contain a minimum of 10,000 bacteria per gram of tissue.18 Despite the presence of acid-fast bacilli in microscopic slides, 6 specimens were PCR-negative, for an overall sensitivity of 71.5%. This finding is consistent with other studies on formalin-fixed, paraffin-embedded tissues19 and might be due to nucleic acid fragmentation secondary to formalin fixation.20 Another cause of false negativity would be nonspecific product formation, especially when the target concentration is low and background DNA is high. These nonspecific products, which are produced by the process of mispriming or primer dimerization, compete with the target during amplification and decrease the efficiency of reaction.21 Mispriming also complicates the evaluation of melting curves by producing nonspecific, wide melting peaks. Although the aforementioned extraction method has been shown to be efficient,16 multiple phases of boiling and freezing leave a substantial amount of single-stranded background DNA, which would increase the probability of mispriming.21 Therefore, using a high-efficiency hot-start polymerase is mandatory. Meanwhile, touch-down PCR and standardization of the background DNA concentration are additional strategies for pushing the reaction in favor of target amplification and escalating the overall sensitivity of the assay.22,23

While prognosis for BCG lymphadenitis is good, in patients with disseminated BCG infection, the outcome is often poor. The histomorphologic pattern might be related to the type of immunodeficiency disorder and clinical outcome.24,25 HIV infection is reported by researchers as the most common predisposing factor for postvaccination BCG infection in South Africa.26 Although primary in contrast with secondary immunodeficiency was more common in the current series, the increase of HIV infection may change this pattern in future. Moreover, when acid-fast bacilli are observed, the infectious agent may be BCG, M tuberculosis, or nontuberculous mycobacteria. Determination of species is clinically important because of different treatment schemes. Meanwhile, clinical management of the disease is difficult, and rapid diagnosis is mandatory.26 Molecular methods can be used for rapid and accurate diagnosis.

References


