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ORIGINAL ARTICLE

Establishment of a new immunological method for direct detection of *Mycobacterium* in solution

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Background/Purpose: Tuberculosis (TB) is a crucial health problem. Prevention of the disease requires rapid diagnosis. Rapid liquid culture systems, nucleic acid amplification tests, and high-performance liquid chromatography (HPLC) are among the rapid tests used for detecting *Mycobacterium* species. However, these tests are expensive and require extensive equipment and expertise, which is hardly affordable in resource-poor countries. Although direct microscopy is performed routinely as an initial step for detection of the bacteria, it is not sufficiently sensitive. As a result, we thought of establishing a low-cost immunological test that can potentially replace direct microscopy with higher sensitivity and specificity.
Methods: The assay is based on pre-incubation of biotinylated rabbit antibody against Antigen 60 (A60) with a solution containing *Bacillus* Calmette-Guérin (BCG) or *Mycobacterium tuberculosis* (MTB) followed by incubation with a streptavidin—alkaline phosphatase (STA—ALP) conjugate. The test is devised in enzyme-linked immunosorbent assay (ELISA) and non-ELISA formats, therefore it does not require extensive facilities and expertise.
Results: The ELISA format showed a 100-fold improvement in the lower detection limit of BCG compared with direct microscopy. With the non-ELISA formats, there was a 2- and 16-fold improvement for the cartridge assay and the microfuge tube assay, respectively.
Conclusion: In conclusion, we successfully detected BCG and MTB in solution using the new immunological method. Our results are very promising and the new immunological method could potentially replace direct microscopy with higher sensitivity and specificity.

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Introduction

Tuberculosis (TB) is a crucial health problem worldwide, based on a report from the World Health Organization (WHO), with 8.7 million cases in 2011.[1] To prevent further transmission and prompt treatment, *Mycobacterium tuberculosis* (MTB) species must be identified rapidly and specifically. New tests including nucleic acid amplification tests for rapid diagnostics of TB infection have been established.[2] Nevertheless, due to the considerable cost of laboratory equipment and the skills required to perform these tests, these methods have been rarely used in third-world countries where TB has the highest prevalence. Among the rapid diagnostic tests for the detection of pulmonary TB, direct microscopy of acid-fast bacteria (AFB) using the Ziehl-Neelsen (ZN) staining method remains the most rapid and low-cost technique and often the best option in developing countries.[2] However, only 40–50% of patients with pulmonary TB are smear-positive[3] and ironically about 17% of TB transmission is through patients with smear-negative and culture-positive TB.[4] By contrast, the Centers for Disease Control (CDC) recommends that 3 consecutive days of sputum collection is needed for initial diagnosis of TB; in developing countries, due to the high workload, laboratory technicians have to prepare a large number of slides every day resulting in a lower quality diagnostic service.[5] The challenge is to develop a simple and inexpensive test with more sensitivity and specificity than that of direct microscopy that could be widely used in resource-poor countries.

In this study, we have developed an assay that could be a potential candidate to replace direct microscopy for detecting various TB species with high sensitivity and specificity. The assay is based on detection of *Bacillus* Calmette-Guérin (BCG) or MTB using a specific biotinylated rabbit polyclonal antibody against Antigen 60 (A60) and a streptavidin–alkaline phosphatase (STA–ALP) conjugate.

The sensitivity of detecting antibodies against MTB A60 in patients with active pulmonary TB is very high, as reported in many published studies.[6–9] Our results are very promising, and the assay may be applicable to detect a wide variety of MTB species in sputum and other body fluids.

Methods

Reagents and materials

BCG was purchased as a BCG vaccine from the Pasteur Institute of Iran (Tehran, Iran). Polyclonal rabbit antibody against A60 and the test cartridges were gifts from Anda Biologicals Company (Strasbourg, France). Antibody biotinylation was performed using Immunoprobe Biotinylation Kit (catalog no. BK-101) purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Nitrophenyl Phosphate (pNPP) and Avidin was purchased from Pierce (Rockford, IL, USA) and Tween-20 from Bio-Rad Laboratories (Hercules, CA, USA). Diethanolamine (DEA), bovine serum albumin (BSA), STA–ALP, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nunc-Immuno™ MicroWell was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sephadex G-25 pre-packed column was purchased from Amersham Biosciences (Uppsala, Sweden).

ZN staining

Different concentrations of BCG preserved in phosphate-buffered saline (PBS) were stained using the ZN staining method. According to the following reporting scale: ZN−, no AFB per 300 fields; ZN±, 1–9 AFB per 100 fields; ZN1+, 10–99 AFB per 100 fields; ZN2+, 1–10 AFB per field in at least 50 fields; and ZN3+, >10 AFB per field in at least 20 fields.

Antibody purification

The antibody against A60 was desalted by a gel filtration step using a Sephadex G-25 column.

Antibody biotinylation

Protein labeling was performed using Immunoprobe Biotinylation Kit following the manufacturer's instructions. Briefly, the content of one vial of biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester (BAC-Sulfo NHS) was dissolved with 30 μL of DMSO, then 0.1M sodium phosphate buffer (pH = 7.2) was added to prepare a final volume of 0.5 mL. The resulting concentration of the BAC-Sulfo NHS solution was 10 mg/mL. Immediately, 38 μL of the BAC-Sulfo NHS solution was added to 1.0 mL of the antibody solution and incubated with gentle stirring for 30 minutes at room temperature (RT). Then the reacting mixture was applied to the gel filtration column to isolate the labeled antibody and 0.5 mL fractions were collected. The highest protein concentration was found in Fraction 4 by measuring absorbance at 280 nm. The test was devised in three formats: capture ELISA, microfuge tube assay, and cartridge assay.

Capture ELISA for BCG

Various concentrations of BCG were diluted in a volume of 300 μL PBS containing 2 mg/mL BSA and 0.01% Tween-20. The microfuge tubes containing diluted BCG were prepared in three series. One series was pre-incubated with 1 μg/mL (1:20), the second series with a dilution of 1/100 biotinylated antibody, and the third series with BCG only. Pre-incubation was at 37°C for 1 hour with gentle stirring. The tubes were then subjected to three washes. To wash the contents of the tubes, they were centrifuged at 10,000 × *g* for 3 minutes, the supernatant was discarded, and the pellet was resuspended in the same buffer.

The ELISA plates were coated with 10 μg/mL STA in acetate buffer (0.01M, pH 5.5) overnight at 4°C. The plates were blocked with 1% BSA in PBS for 1 hour at RT, and then washed three times with washing solution. The contents of the tubes were applied to the ELISA plates, and incubated for 50 minutes at RT. Five washes were carried out on the plates and then they were incubated with STA–ALP in 50 mM Tris buffer, pH 7.5 for 20 minutes at RT. Finally, the plates were washed four times with 0.1% Tween-20 solution