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

in distilled water, and 100 μL of a solution of 2 mg/mL *p*-Nitrophenyl Phosphate (pNPP) substrate in 50 mM Dieth-anolamin (DEA) buffer containing 1 mM $MgCl_2$ was added to each well and left at RT for 30 minutes.

Capture ELISA for MTB

The MTB bacilli were obtained from clinical isolates and inactivated in a water bath at 80°C for 20 minutes. According to our institutional ethical board, informed consent was not necessary because samples were already collected for other purposes. Two concentrations of bacilli (1.5×10^4 bacilli/mL and 1.5×10^5 bacilli/mL) were diluted in a volume of 300 μL PBS containing 2 mg/mL BSA and 0.01% Tween-20. These di-lutions were then pre-incubated with a 1:50 dilution of bio-tinylated antibody in the medium at 37°C for 1 hour with gentle stirring. Tubes were subjected to three subsequent washes. To wash the contents of the tubes, they were centrifuged at $10,000 \times g$ for 5 minutes, the supernatant was discarded, and the pellet was re-suspended in the same buffer. The following steps were conducted exactly as was described for the capture ELISA for BCG.

Microfuge tube assay

Various concentrations of BCG were diluted in a volume of 500 μL PBS containing 2 mg/mL BSA and 1% Tween-20. The tubes containing diluted BCG were pre-incubated with 1:50 biotinylated antibody at 37°C for 1 hour with gentle stir-ring. Tubes were subjected to three washes. To wash the tubes, they were centrifuged at $10,000 \times g$ for 3 minutes, the supernatant was discarded, and the pellet was sus-pended in 50 mM Tris buffer containing 2 mg/mL BSA and 0.01% Tween-20. After the last wash, the pellet was sus-pended in 100 μL of the STA—ALP conjugate at RT for 20 minutes. Following three more washes using 0.1% Tween-20 solution, tubes were incubated with 200 μL pNPP and the optical density (OD) was measured after 30 minutes.

Cartridge assay

The pre-incubation steps were similar to that of the microfuge tube assay. The contents of the pre-incubated tubes with biotinylated antibody were applied to the car-tridges, and then there were three washes using 1% Tween-20 solution. In this step, unbound antibodies and other small molecules were filtrated through the cartridge filter, and only the antibodies bound to the bacilli were retained by the filter. The STA—ALP conjugate (100 μL) was then added to the cartridge filter, and after three washes 200 μL of pNPP was added. Finally, photography was carried out after 10 minutes. Fig. 1 depicts the steps involved in the experiments.

Results

Capture ELISA for BCG

Using the newly established capture ELISA, we determined ODs at lower concentrations of BCG compared with the ZN

staining method (Fig. 2). The sensitivity of the capture ELISA was 100-fold higher than ZN direct microscopy considering the lower detection limit of 15,000 bacilli/mL in ZN staining and 150 bacilli/mL in ELISA. According to the figure, using a lower dilution (higher concentration) of biotinylated antibody resulted in higher ODs with the same BCG concentrations. To ensure that ODs were not due to nonspecific bindings of antibody molecules to the ELISA plates, we measured the absorbance of the antibody without BCG and BCG without antibody and found these to be insignificant.

Capture ELISA for MTB

Using the newly established capture ELISA, we compared ODs at two concentrations of MTB. At the lowest concen-tration, we detected the lowest detection limit of MTB with the ZN staining method (1.5×10^4 bacilli/mL; Fig. 3).

Microfuge tube assay

Using the microfuge tube assay, we measured the ODs of very low BCG concentrations that did not stain positive with the ZN method (Fig. 4). Owing to the novel microfuge tube assay, it was possible to detect BCG concentrations as little as 1000 bacilli/mL approximately.

Cartridge assay

Fig. 5 shows the response of serially diluted BCG ($0-1.5 \times 10^5$ bacilli/mL). The yellow color represents a positive reaction. Based on the figure, using lower antibody dilutions (1:20) resulted in positive reactions at lower BCG concentrations, i.e., 1.5×10^4 bacilli/mL with 1:20 anti-body compared to 1.5×10^5 bacilli/mL with 1:100 antibody. However, with the naked eye we observed a weak positive reaction at a BCG concentration of 7.5×10^3 bacilli/mL using 1:20 antibody, which is not easily visible in the photograph.

Fig. 6 shows a comparison of the lower detection limits of the three test formats.

Discussion

TB is a crucial health problem and controlling the disease requires rapid and accurate diagnosis. However, ZN direct microscopy as the most common rapid test used in resource-poor countries where TB has the highest incidence is not sufficiently sensitive and specific.[2]

The methods proposed here are both patient and technician friendly. Only one sputum sample is required at the first visit and a large number of specimens can be handled simultaneously. Additionally, expensive equip-ment and materials are not required to perform these rapid tests. Although the new assay may seem more expensive than direct microscopy at a glance, we believe it is more cost-effective than the conventional direct microscopy method considering the cost of repetitive sampling and culturing sputum for negative cases. A cost-effective study is needed to explore our idea.[10]
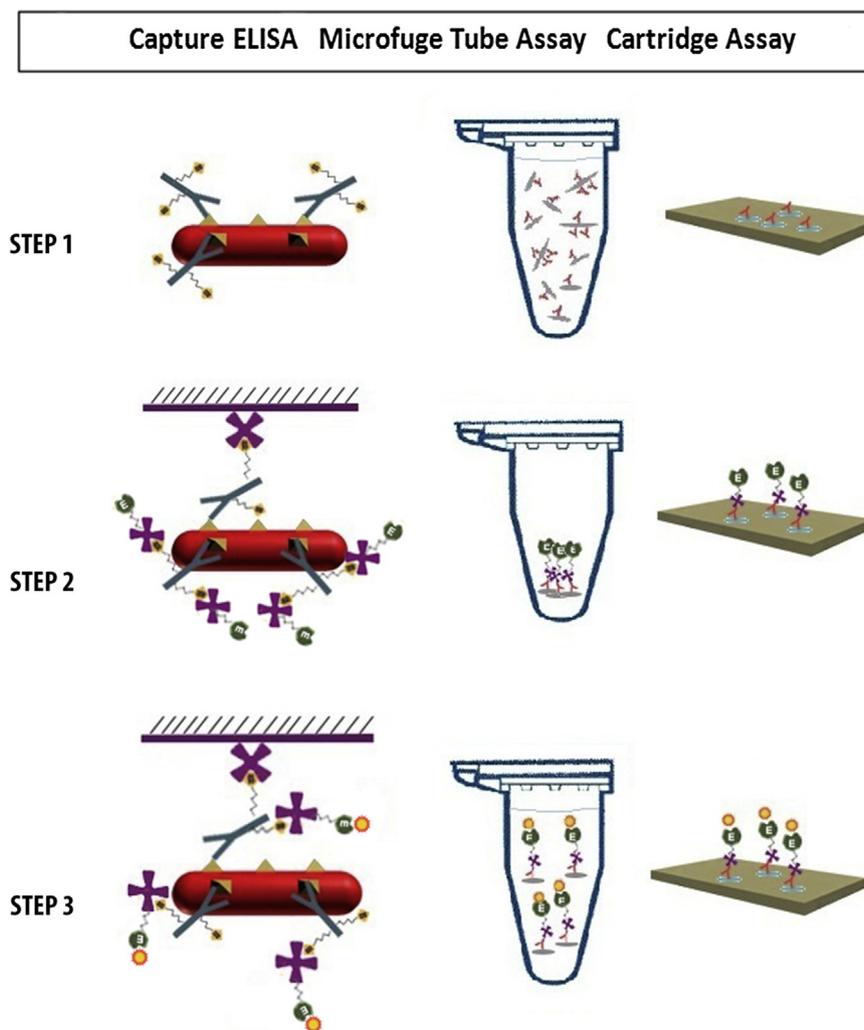
Capture ELISA   Microfuge Tube Assay   Cartridge Assay

Figure 1. Illustrated scheme of the three assay formats. Each assay format has three steps. Step 1 represents pre-incubated bacilli (or BCG) with biotinylated antibody. The biotin molecules are not shown in the microfuge tube assay and the cartridge assay. Step 2 represents the addition of the STA—ALP conjugate. Step 3 represents addition of the substrate. BCG = *Bacillus* Calmette-Guérin; STA—ALP = streptavidin—alkaline phosphatase.

Chemically, the assay is based on pre-incubation of biotinylated rabbit antibody against A60 with a sample containing BCG or MTB followed by incubation with a STA—ALP conjugate. In all immunochemical assays, capture of analytes using antibodies requires long incubation to obtain a good response; however, this is not always feasible due to high nonspecific binding of antibody to the solid support in ELISA formats. In the present work, we circumvented this problem through the pre-incubation approach in a tube, prior to adding to the ELISA wells.[11] The second step of the assay took advantage of rapid capture of the biotinylated antibody with an STA enzyme conjugate with a very high affinity ($K_d = 10^{-15}$). To our knowledge, such use of biotin—avidin chemistry to detect microbial cells is novel and it may be employed to detect other pathogens in clinical specimens or microbial cultures.

Although there are more sensitive antigens for serodiagnosis of TB,[12–15] we simply wanted to display the feasibility of the technique; we have probably not selected the best antigen for TB serodiagnosis, and this was due to the unavailability of other antibodies.

Such use of the biotin—avidin system improved the lower detection limit 100-fold in the capture ELISA for BCG as illustrated in Fig. 2. The higher sensitivity compared to direct microscopy was due to the highly specific interactions of avidin—biotin molecules. Moreover, because more than one A60 exists on a single bacterium (Fig. 1), more than one biotinylated antibody binds to the antigen resulting in signal amplification. Furthermore, we circumvented the problem of low signal-to-noise ratio owing to the pre-incubation method. To ensure that the resulting response was not due to nonspecific bindings of the antibody molecules to the ELISA plates, we measured the OD of the antibody without BCG and BCG without antibody and found these to be unnoticeable.

In the capture ELISA for MTB, we determined a lower detection limit of 1.5×10^4 bacilli/mL similar to ZN direct microscopy. Although there was no improvement compared to the ZN method, we wanted to show that the test was also
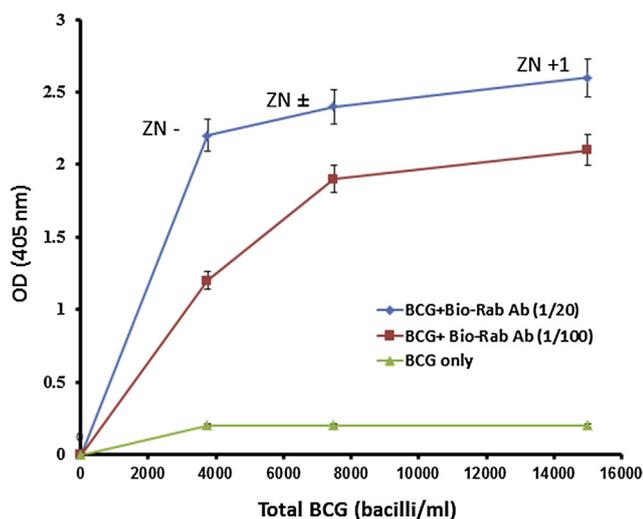
Figure 2. The results of the capture assay using BCG as the analyte. The ODs of various concentrations of BCG (bacilli/mL) after performing the test with two different dilutions of biotinylated rabbit antibody. The ZN staining result for each BCG concentration is shown on the graph. BCG = *Bacillus* Calmette-Guérin; Bio-Rab Ab = biotinylated rabbit polyclonal antibody; OD = optical density; ZN = Ziehl-Neelsen.

applicable for MTB. Such lack of improvement is probably because A60 does not expose in MTB in a way that it does in BCG. Furthermore, there is evidence that A60 proportions vary depending on the growth stage of the bacteria. A60 from BCG contains equal amounts of protein, lipids, and carbohydrates during the stationary phase of growth, but the protein content can increase to be seven times higher than that of polysaccharides in the exponential phase of growth.[16] Similarly, this could be true of MTB resulting in incongruity between the results obtained from BCG and MTB assays. We believe that the method will be more suitable for MTB detection if antibodies against more
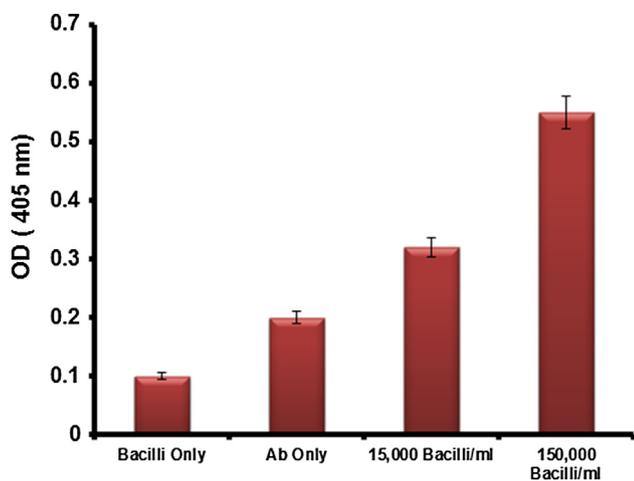
sensitive antigens such as lipoarabinomannan (LAM) and Early Secreted Antigen Target-6 ESAT-6[12—15] are used.

The capture ELISA format showed a very high sensitivity detecting as little as 150 bacilli/mL of BCG. The microfuge format showed a moderately high sensitivity detecting approximately 1000 bacilli/mL of BCG. Finally, the cartridge assay showed an acceptable sensitivity detecting 7.5×10^3 bacilli/mL of BCG. In other words, the ELISA format, the microfuge format, and the cartridge assay resulted in 100-, 16-, and 2-fold improvement, respectively, in the lower detection limit of BCG when compared with direct microscopy (i.e., 1.5×10^4 bacilli/mL of BCG) (Fig. 6).

Additionally, the microfuge tube format did not require ELISA plates and an ELISA reader to perform the test. Moreover, because the cartridge assay did not require extensive equipment and expertise to carry out, it can be considered as a point-of-care test (POCT). If the cartridge assay is done using monoclonal antibodies rather than polyclonal ones, it may lead to a lower detection limit.

All three assay formats were practically faster and more sensitive than ZN direct microscopy. For high quality smear examination, the technical guide of the International Union against Tuberculosis recommends that each laboratory technician should not process and read more than 25 ZN-stained sputum specimens per day, and that no more than 10—12 specimens should be processed at one time. The guide recommends that technicians should take at least 5 minutes to examine a slide. Therefore, considering these recommendations and an average 15-minute time for smear preparation, every 12 specimens takes around 1 hour and 15 minutes to be processed and examined thoroughly, taking about 3 hours per day per technician for 25 specimens. On comparison, the ELISA,
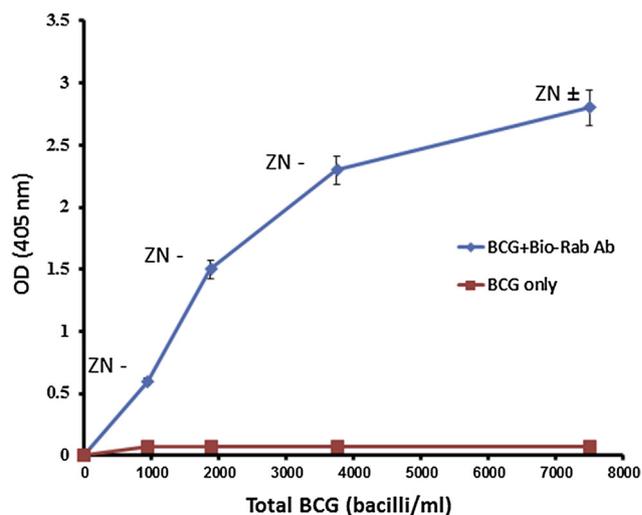

Figure 3. The results of the capture assay using bacilli as analytes. The ODs of two MTB concentrations are displayed. The OD of bacilli without antibody and the OD of antibody alone are shown. MTB = *Mycobacterium tuberculosis*; OD = optical density.

Figure 4. The results of the microfuge tube assay using BCG as analyte. The ODs of various concentrations of BCG (bacilli/ mL) detected with biotinylated rabbit antibody. The ZN staining result for each BCG concentration is shown on the graph. BCG = *Bacillus* Calmette-Guérin; Bio-Rab Ab = biotinylated rabbit polyclonal antibody; OD = optical density; ZN = Ziehl-Neelsen.

Figure 5. The results of the cartridge assay at different BCG and antibody concentrations. The numbers 0, 1, 2, 3, 4, and 5 represent 0 bacilli/mL, 7,500 bacilli/mL, 15,000 bacilli/mL, 30,000 bacilli/mL, 60,000 bacilli/mL, and 120,000 bacilli/mL of BCG. The test was done with 1:100 antibody dilution (upper row) and 1:20 antibody dilution (lower row). BCG = *Bacillus* Calmette-Guérin.
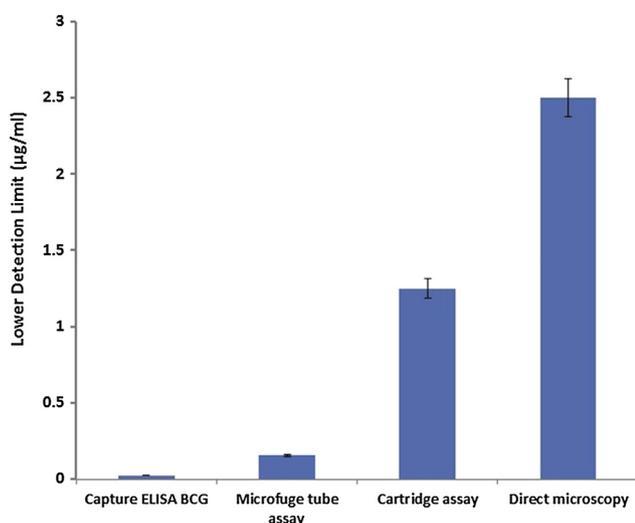

Figure 6. Comparison of the lower limit of detection of the three test formats with the ZN direct microscopy method. BCG = *Bacillus* Calmette-Guérin; ELISA = enzyme-linked immunosorbent assay; ZN = Ziehl-Neelsen.

microfuge, and cartridge assay proposed here takes about 2 hours to be completed thoroughly, and the method could be applied to 96-well ELISA plate and no less than 25 specimens simultaneously (compared to 25 ZN-stained specimens).

In conclusion, our results are very promising in terms of improving sensitivity and cost for detection of various *Mycobacterium* species. The assay needs further evaluation using antibodies against specific antigens for direct diagnosis of MTB and also other pathogens in clinical samples.

Conflicts of interest

The authors have no conflicts of interest to declare.

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