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Rapid diagnosis of tuberculosis by identification of Antigen 85 in mycobacterial culture system☆

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ABSTRACT

The standard culture for identification of *Mycobacterium tuberculosis* takes a long time to perform. We introduce here a method for fast identification of *M. tuberculosis* in mycobacterial culture system. Antibodies to Antigen (Ag) 85 of *M. tuberculosis* were produced and subsequently used to develop enzyme-linked immunosorbent assay (ELISA) for detecting Ag85 in the culture filtrate. By this detection, rapid tuberculosis (TB) diagnosis was achieved in comparison to the standard culture system with 89.6% sensitivity and 94% specificity. We thus suggest a new TB diagnosis strategy in which clinical samples are cultured in mycobacteria liquid culture medium. The culture filtrates are taken for detection of the Ag85 by ELISA. Using this strategy, 25%, 50%, 80%, and 90% of TB patients will be detected within day 3, week 1, 2, and 4, respectively. The established assay will enable a faster diagnosis of TB, leading to more efficient treatment of TB patients and control of disease transmission.

1. Introduction

Tuberculosis (TB) is one of the world's most serious infectious diseases. One third of the world's population is assumed to be infected with *Mycobacterium tuberculosis* complex, the causative agent of TB (Lauzardo and Ashkin, 2000). Because its occurrence is in association with the human immunodeficiency virus (HIV), the prevalence of TB is currently increasing, especially in HIV-endemic countries (Narayanan et al., 2003). Rapid and accurate diagnosis of TB, therefore, is a very important component of world health measures (Kashyap et al., 2007a; Parsons et al., 2011). Fast diagnosis of TB can lead to appropriate initiation of treatment of TB patients and control of disease transmission in the community. The methods routinely performed in hospitals and health care units for diagnosis of TB include acid-fast bacilli (AFB) staining and mycobacterial culture (Parsons et al., 2011). However, these TB diagnostic methods have several disadvantages. The sensitivity of a direct smear for AFB is very poor, leading to incorrect diagnosis (Brodie and Schluger, 2005). Culturing of the etiologic agent is currently accepted as the standard method for the diagnosis of TB. However, this method takes a very long time, i.e., 6–8 weeks, in order to demonstrate the growth of *M. tuberculosis* (Kashyap et al., 2007b; Negi et al., 2006; Rattan et al., 1994; Selvakumar et al., 2002). The culture results are often obtained too late to affect initial disease management (Parry, 1993). Recently, several methods for diagnosing TB have been developed, such as fluorescent antibody test, DNA hybridization, and polymerase chain reaction (PCR) (Haldar et al., 2011; Woods, 2001). However, all require a well-established laboratory, costly equipment, and highly trained personnel (Nikam et al., 2013; Palomino, 2005; Woods, 2001). These methods are not appropriate for use in resource-limited countries. A quick, economical, sensitive, and specific diagnostic test for TB is, therefore, desirable for facilitating early treatment (Lalvani et al., 2001).

Antigen (Ag) 85 complex is the major secretory proteins of *M. tuberculosis* (Wiker and Harboe, 1992). The Ag85 complex comprises 3 related proteins, Ag85A (32 kDa), Ag85B (30 kDa), and Ag85C (32.5 kDa). The Ag85 complex possesses enzymatic mycolyltransferase and plays a vital role in the physiology of the mycobacterium (Anderson et al., 2001). Among the Ag85 complex, Ag85B has been demonstrated to be the major secreted protein (Harth et al., 1996). The Ag85 proteins can be detected in the sputum of pulmonary TB patients and cerebrospinal fluid of TB meningitis patients (Kashyap et al., 2007b) and have been suggested as markers for TB diagnosis (Malen et al., 2008). In the present study, monoclonal and polyclonal antibodies to Ag85B were produced and applied for development of

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an enzyme-linked immunosorbent assay (ELISA) for detection of the Ag85 complex. The developed ELISA enabled rapid detection of the Ag85 protein present in mycobacterial culture filtrates, thus allowing rapid diagnosis of TB.

2. Materials and methods

2.1. The recombinant Ag85B production

The PCR was performed to amplify the Ag85B coding sequence using *M. tuberculosis* H37Rv DNA as a template. The primers used were forward primer 5′GAGGAGGAGGTCATATGTTCTCCC-GGCCGGGGCT 3′ that has a specific cutting site for *Nde*I and reverse primer 5′GAGGAGGAGCTGAATTCGCCGGCGCCTAACGAACTCT 3′, which has a specific cutting site for *Eco*RI. The PCR cycling conditions started at 1 cycle at 95 °C for 5 minutes followed by 34 cycles of denaturation at 94 °C for 50 seconds, annealing at 53 °C for 50 seconds, and extension at 72 °C for 1 minute. After 35 amplification cycles, the mixture was kept at 72 °C for 10 minutes. The Ag85B gene was then ligated into pAK400CB vector (a kind gift from Dr Ville Santala, University of Turku, Finland) (Tayapiwatana et al., 2006) and named pAK400CB-Ag85B. The obtained vector was transformed into *Escherichia coli*. The correct insertion of the Ag85B gene in the vector was verified by restriction enzyme analysis, PCR, and DNA sequencing. The clone of *E. coli* containing the pAK400CB-Ag85B plasmid was cultured in super broth medium containing biotin (Sigma-Aldrich, St Louis, MO, USA), and isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich) was added to induce protein expression. Under these conditions, the produced Ag85B proteins were expressed as the biotinylated form (Tayapiwatana et al., 2006). The recombinant proteins were extracted from the bacterial pellet using B-PER II extracting reagent (Thermo Scientific Pierce, Rockford, IL, USA). The recombinant Ag85B protein from the bacterial extract was purified using streptactin Sepharose affinity chromatography. The bacterial extract was rotated with streptactin beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) at 4 °C for 18–20 h. Beads were collected by centrifugation at 5000 g at 4 °C for 5 minutes. The bound proteins were then eluted by rotation of beads in elution buffer (2.5 mmol/L des-thiobiotin in phosphate-buffered saline [PBS] pH 7.2) at room temperature for 20 minutes. The eluted proteins were concentrated using an Amicon Ultra centrifugal filter with a 10-kDa molecular weight cutoff (Sigma-Aldrich). The concentrated proteins were dialyzed against PBS pH 7.2 and determined for protein concentration using a BCA kit (Thermo Scientific Pierce). The purity of the obtained protein was verified by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel.

2.2. Detection of the recombinant biotinylated-Ag85B proteins by ELISA

The ELISA plate was coated with 50 μL of 10 μg/mL avidin (Sigma-Aldrich) in carbonate/bicarbonate buffer pH 9.6 at 4 °C for 18 h and blocked with blocking buffer (2% skimmed milk in PBS, pH 7.2) for 1 h at 37 °C. The ELISA plate was washed 4 times with washing buffer (0.05% Tween 20 in PBS, pH 7.2). Fifty microliters of the bacterial extract containing biotinylated-Ag85B or biotinylated-CD147 recombinant proteins (control) (generated in our laboratory) was added into each well of the ELISA plate and incubated at 37 °C for 1 h. After washing 4 times, 50 μL of the rabbit polyclonal antibody (pAb) to Ag85B (Abcam, Cambridge, UK) was added. The plate was incubated 1 h at 37 °C and washed 4 times. To detect antigen-antibody complexes, horseradish peroxidase (HRP) conjugated swine anti-rabbit immunoglobulin antibodies (Dako, Glostrup, Denmark) were added and kept at room temperature for 1 h. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Invitrogen, Camarillo, CA, USA) was added into each well. After 15 minutes, the reaction was stopped by 1 N HCl, and the absorbance was read at 450 nm.

2.3. Monoclonal antibody production

Bead immunization strategy was employed for induction of antibody responses (Pata et al., 2009; Tayapiwatana et al., 2006). The recombinant biotinylated-Ag85B proteins were isolated from bacterial lysate using streptavidin-coated magnetic particles (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The Ag85-captured beads were directly used for mouse immunization. A BALB/c mouse was intraperitoneally immunized with Ag85B-caputred beads 3 times at 2-week intervals. After reaching a high antibody titer, spleen cells from the immunized mouse were fused with myeloma cells (P3-X63Ag8.653) using a standard hybridoma technique (Pata et al., 2009; Tayapiwatana et al., 2006). Hybridomas producing anti-Ag85B antibodies were determined by indirect ELISA. Large scale production of anti-Ag85B monoclonal antibody (mAb) was achieved by cultivation of hybridoma cells in serum-free media (Gibco, Grand Island, NY, USA) and purified by affinity chromatography using a protein G Sepharose column (GE Healthcare Bio-Sciences).

2.4. Polyclonal antibody production

A rabbit was subcutaneously immunized with 1 mg of bacterial extract containing recombinant Ag85B protein at 2-week intervals 3 times. Freund's Complete Adjuvant (Sigma-Aldrich) was used in the first immunization, and Freund's Incomplete Adjuvant (Sigma-Aldrich) was used in the second and the third immunizations. Blood samples were collected, and sera were separated. Anti-Ag85B antibody titer was determined by indirect ELISA. The immunoglobulin fraction was purified from the serum using a protein G Sepharose column (GE Healthcare Bio-Sciences).

2.5. Detection of the anti-Ag85B antibody by ELISA

The ELISA plate was coated with 50 μL of the 10 μg/mL avidin (Sigma-Aldrich) in carbonate/bicarbonate buffer pH 9.6 at 4 °C for 18 h. The bacterial extract containing biotinylated-Ag85B or biotinylated-CD147 recombinant proteins (control) was added into each well and incubated for 1 h at 37 °C. After being washed 4 times, 50 μL of the tested serum or culture supernatants was added into each well and incubated for 1 h at 37 °C. To detect antigen-antibody complexes, HRP conjugated rabbit anti-mouse immunoglobulin antibodies (Dako) were added into each well. The ELISA plate was kept at room temperature for 1 h and washed 4 times. TMB substrate (Invitrogen) was added into each well and incubated for 15 minutes, the reaction was stopped by 1 N HCl, and the absorbance was read at 450 nm.

2.6. Determination of the activity of anti-Ag85B monoclonal antibody by Western immunoblotting

To prepare native Ag85 protein, *M. tuberculosis* strain H37Rv was cultured in protein-free Sauton medium at 37 °C for 4–6 weeks until the turbidity was equivalent to the McFarland nephelometer standard number 1. The supernatant was collected and filtered through a 0.2-μm membrane filter (Pall, Ann Arbor, MI, USA) and then concentrated using an Amicon Ultra centrifugal filter with a 10-kDa molecular weight cutoff (Sigma-Aldrich). The obtained culture filtrate containing Ag85 was measured for total amount of protein with a BCA kit (Thermo Scientific Pierce) and stored at −70 °C until use.

Recombinant Ag85B and native Ag85 protein were separated by SDS-PAGE on 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% skimmed milk at 4 °C for 18 h. The membrane strips were incubated with the tested antibodies for 1 h at room temperature on a shaking platform. After washing for 3 times with 0.1% Tween 20 in PBS (pH 7.2), membrane strips were incubated with HRP conjugates for 1 h at room temperature on a shaking platform. The membrane strips were then