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Enzyme-linked immunospot assay response to recombinant CFP-10/ESAT-6 fusion protein among patients with spinal tuberculosis: implications for diagnosis and monitoring of surgical therapy

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SUMMARY

Objective: This study aimed to assess the performance of a laboratory-developed recombinant CFP-10/ESAT-6 fusion protein (rCFP-10/ESAT-6)-based enzyme-linked immunospot (ELISPOT) assay for the diagnosis of spinal tuberculosis (TB) in China, and to evaluate the value of the ELISPOT assay for monitoring the efficacy of surgical treatment.
Methods: In the first part of the study, a total of 78 participants were consecutively recruited for ELISPOT using rCFP-10/ESAT-6 as a stimulus. The cutoff value for ELISPOT positivity was based on the results of receiver operating characteristic curve analysis. In the second part, this approach was evaluated in a prospective study including 102 patients with suspected spinal TB. Data on clinical characteristics of the patients and conventional laboratory results were collected, and blood samples were obtained for ELISPOT using rCFP-10/ESAT-6 as a stimulus.
Results: Among the 102 patients with suspected spinal TB, 11 were excluded from the study. Twenty-three patients (25.2%) had culture-confirmed TB and 29 (31.9%) patients had probable TB. Among the spinal TB patients, the ELISPOT had a sensitivity of 82.7%, compared to a sensitivity of 61.5% for the purified protein derivative (PPD) skin test. The specificity was 87.2% for ELISPOT and 46.2% for the PPD skin test among 39 subjects with non-TB disease. The number of spot-forming cells and/or the positive rate of the ELISPOT assay were associated with aging, emaciation, and paravertebral abscess. The number of subjects with responses to rCFP-10/ESAT-6 slightly decreased after surgical treatment in spinal TB patients.
Conclusions: A laboratory-developed rCFP-10/ESAT-6 ELISPOT assay is a useful adjunct to current tests for the diagnosis of spinal TB.

1. Introduction

Spinal tuberculosis (TB) is found in 1–3% of all TB cases and in 50–60% of cases of musculoskeletal TB.[1] However, the diagnosis of spinal TB is difficult to set, not only because of its nonspecific clinical presentation, but also because of the lack of useful diagnostic tests. Diagnostic methods used for spinal TB are tuberculin skin testing, radiographic image examination, culture, and PCR assays. Tuberculin skin testing is a conventional test with a sensitivity ranging from 67% to 72%, but it cannot differentiate between active and past infections and can also be positive due to bacille Calmette–Guérin (BCG) vaccination.[2] Most importantly, confirmation testing by mycobacterial culture is time-consuming and often takes weeks to complete. Thus, there is a need for a rapid and accurate diagnostic test.

An in vitro T-SPOT-TB assay using pools of early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) peptides, has recently been manufactured and commercialized (Oxford Immunotec, Oxford, UK).[3] It is based on the detection of interferon-gamma (IFN-γ) released by activated T lymphocytes. The stimuli used, ESAT-6 and CFP-10 peptides, are located within region of difference 1 (RD1) of the *Mycobacterium tuberculosis* and *Mycobacterium bovis* genomes, but is absent from all strains of *M. bovis* BCG, as well as from most non-tuberculous mycobacteria (NTM).[4–6] This assay has been demonstrated to provide useful support in the diagnosis of skeletal TB.[7,8] However, the use of peptides increases the cost of the kit, making it unaffordable in developing countries. A fusion protein as the stimulus would offer

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a cheaper and more realistic alternative for large-scale production and clinical use in developing countries. A laboratory-developed recombinant CFP-10/ESAT-6 fusion protein (rCFP-10/ESAT-6)-based enzyme-linked immunospot (ELISPOT) assay has been demonstrated to be useful for the diagnosis of active pulmonary TB in China.[9] The aim of this study was to assess the diagnostic value of a laboratory-developed rCFP-10/ESAT-6 ELISPOT assay in clinical cases of spinal TB in China. In subgroups of subjects, we repeated the assay before and after surgical treatment to assess the evolution of responses. To our knowledge, this is the first clinical evaluation of rCFP-10/ESAT-6 as the stimulus in an ELISPOT assay for the diagnosis of spinal TB and for monitoring the efficacy of surgical treatment.

2. Materials and methods

2.1. Participants and study design

Participants were consecutively recruited at two hospitals (Nanfang Hospital, Southern Medical University, an 1800-bed medical center in southern China; Guangzhou Thoracic Hospital, a 600-bed medical center in southern China) from May 2011 to September 2012. Ethical approval for the study was granted by the ethics committee of Nanfang Hospital.

In phase 1, 78 participants were consecutively enrolled into the study. Patients were untreated or had received <2 weeks of anti-TB therapy at the time of venipuncture for the ELISPOT assay. Fifty cases had a final diagnosis determined according to the following criteria: patients classified as having confirmed TB were those with clinical specimens positive for *M. tuberculosis* on culture; patients were classified as having probable TB if histological findings of a biopsy specimen were consistent with a diagnosis of TB infection (granulomatous inflammation and/or caseating necrosis) and if they responded clinically and radiologically to a full course of anti-TB treatment according to the criteria described in previous studies;[10,11] patients were classified as not having TB if another diagnosis was made or if there was clinical improvement without anti-TB therapy. Enrolled participants included 30 spinal TB patients, 20 non-TB patients, and 28 healthy volunteers. Based on the rCFP-10/ESAT-6 ELISPOT results of these participants, receiver operating characteristic curve analysis was performed to determine the optimal cutoff value of the ELISPOT assay.

In phase 2, a total of 102 patients with suspected spinal TB were prospectively recruited during the study period. Data on clinical characteristics of the patients and conventional laboratory results were collected. The specific data collected were age, sex, underlying diseases, radiographic image examination, lymphocyte count, pathology, microbiology results, and follow-up observations. All cases were independently classified by two of the study investigators (QZ, JC) who were blinded to the ELISPOT assay results. Diagnostic criteria were based on the above standard. After a follow-up to September 2012, 11 patients were excluded from the study, among whom five did not complete the ELISPOT assay and six had no final diagnosis. The remaining 91 patients were ultimately included for ELISPOT analyses. The spinal TB group consisted of 52 cases (23 confirmed cases and 29 probable cases), with a mean age of 42.3 ± 18.4 years (range 2–86 years). The non-TB disease group consisted of 39 subjects, with a mean age of 46.5 ± 13.9 years (range 18–69 years). All patients enrolled in the present study were tested for HIV by serology and all had negative results. Details of the 91 patients with suspected spinal TB are shown in Table 1.[12]

Table 1
Epidemiological and demographic characteristics of 91 patients with suspected spinal TB

Variables	Definite TB (n = 52)	Non-TB disease (n = 39)[a]
Age, years, mean ± SD (range)	42.3 ± 18.4 (2–86)	46.5 ± 13.9 (18–69)
Male to female sex ratio	31:21	17:22
Underlying condition or illness (%)		
Hypertension	3 (6)	7 (18)
Pneumonia	1 (2)	2 (5)
Hepatitis B	5 (10)	0
Syphilis	2 (4)	1 (3)
Diabetes mellitus	3 (6)	2 (5)
Immunocompromised condition[b] (%)	10 (19)	2 (5)
Suspected sites of infection (%)		
Cervical vertebra	0	6 (15)
Thoracic vertebra	22 (42)	5 (13)
Thoraco-lumbar	5 (10)	0
Lumbar vertebra	23 (44)	28 (72)
Sacral vertebrae	2 (4)	0
Combined pulmonary TB (%)	7 (13)	2 (5)

TB, tuberculosis; SD, standard deviation. Data are presented as number (%).
[a] Non-TB disease: sixteen patients with lumbar disc herniation; six patients with cervical spondylosis; eight patients with spinal canal tumor; five patients with spinal tumor; four patients with suppurative spondylitis.
[b] Immunocompromised patients were defined as those with underlying diseases such as malignancy, liver cirrhosis, and chronic renal failure, or those receiving immunosuppressive treatment.[12]

2.2. Laboratory procedures and histopathology

Microbiological and pathological specimens for diagnosing spinal TB were processed by standard techniques and procedures. In brief, mycobacteria were cultured on solid culture medium, and the *M. tuberculosis* complex was identified with a commercial DNA probe (AccuProbe Mycobacterium TB complex culture identification kit; GenProbe, San Diego, CA, USA). If the AccuProbe assay was negative, cultures were identified with a commercially available PCR test for NTM (kit for the identification and drug sensitivity testing of non-tuberculous Mycobacterium; Gaoteng, Nanchang, China). For histopathological examination, formalin-fixed and paraffin-embedded tissue blocks of biopsied specimens were stained with hematoxylin–eosin stain. Smears of the decontaminated specimens were stained with the Ziehl–Neelsen stain and examined for acid-fast bacilli (AFB).

2.3. Preparation of rCFP-10/ESAT-6 fusion protein

The fusion protein of CFP-10 (Rv3874) and ESAT-6 (Rv3875) was engineered as described previously.[13–15] In brief, the individual genes were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR. During the amplification steps the genes were fused with a linker encoding glycine–glycine–glycine–glycine–serine–glycine–glycine–glycine–glycine–serine–glycine–glycine–glycine–glycine–serine. The product was subsequently cloned into plasmid vector pET-28a (Novagen, San Diego, CA, USA) containing a C-terminal hexa-histidine tag. Sequencing was performed to confirm the identity of the cloned DNA fragment. The recombinant fusion protein was over-expressed in *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) and was purified by metal chelate column chromatography using nickel–nitrilotriacetic acid (Ni–NTA) resin in accordance with the manufacturer's protocol (Qiagen). Recombinant protein batches were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining and Western blotting with a murine anti-His tag monoclonal antibody (Novagen, San Diego, CA, USA), to confirm the size and purity of the protein.