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DIAGNOSTICS

Development of a time-resolved fluoroimmunoassay of CFP-10 for rapid diagnosis of tuberculous pleural effusion

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SUMMARY

Tuberculous pleural effusion is the second most common form of extrapulmonary tuberculosis, which is very difficult to rapidly distinguish from malignant pleural effusion in the clinical setting. A time-resolved fluoroimmunoassay (TRF) of CFP-10, a low molecular weight protein secreted by pathogenic Mycobacterium tuberculosis, was developed to differentiate tuberculous pleural effusion from malignant one. The measuring range was 0.3–187.5 ng/ml with the dose–response coefficient of 0.9998 and detection limit of 0.036 ng/ml. The intra-assay and inter-assay coefficients of variation were 3.6–9.2% and 10.0–12.4%, respectively. The concentration of CFP-10 in malignant pleural effusion was less than 0.8 ng/ml. The negative predictive value was 93.1% in malignant pleural effusion ($n = 247$) while the positive predictive value was 83.0% in tuberculous pleural effusion ($n = 235$). Moreover, there was a statistically significant difference in the CFP-10 concentration of pleural effusion between the groups before and after clinical therapy of tuberculosis ($P < 0.001$, $n = 81$). In addition, the stability of the diagnostic reagents lasted at least 1 year at 4 °C. Therefore, the TRF of CFP-10 may be used for the rapid diagnosis of tuberculous pleural effusion and further monitoring the clinical therapeutic efficacy of tuberculosis.

1. Introduction

Tuberculosis (TB) results in an estimated 1.7 million deaths each year and the worldwide number of new cases (more than 9 million) is higher than at any other time in history. In many regions highly endemic for TB, the diagnosis continues to rely on century-old sputum microscopy; there is no vaccine with adequate effectiveness and TB treatment regimens are protracted and have a risk of toxic effects. Increasing rates of drug-resistant tuberculosis in Eastern Europe, Asia, and sub-Saharan Africa now threaten to undermine the gains made by worldwide TB control programs [1].

Tuberculous pleural effusion, which results from the infiltration of the pleural space by Mycobacterium tuberculosis antigens or bacilli, is the second most common form of extrapulmonary TB, only less frequent than tuberculous lymph node. With pleural fluid analysis, acid fast bacilli are detected in <5% of cases, whereas mycobacterial culture of fluid has a sensitivity of <60%, with waiting times of up to 2 months for culture results. In addition to mycobacterial culture, current methods used to diagnose tuberculous pleural effusion include measurement of adenosine deaminase (ADA) level, quantification of interferon-γ, nucleic acid amplification test and pleural biopsy. However, the limited specificity or/and sensitivity couldn't meet the clinical requirement for rapid diagnosis of tuberculous pleural effusion [2].

Time-resolved fluoroimmunoassay (TRF) has initiated the development of a new generation of immunoassays by use of lanthanide chelate labels with unique fluorescence properties. In brief, the fluorescence intensity is measured after a selected delay time which almost completely eliminates background fluorescence with a fast decay time. The excitation is performed with a flashing light source. The molecules with a long fluorescent lifetime consist of chelates of rare earth metals (Eu, Tb, Sm, Dy). They absorb strongly the excitation radiation and transfer the energy to the chelated central atom which in turn produces an emission spectrum characteristic of the lanthanide used. A long Stokes' shift (greater than 270 nm) helps to reduce the background in the

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emission region of the chelate and thus optimizes measurement of the relevant fluorescence. In short, key among these properties is a very long fluorescence decay time and an exceptionally large Stokes' shift. Additionally, the label, a lanthanide chelate, is dissociated from the antibody or reporter molecule into a new, highly fluorescent chelate within a protective micelle. Most importantly, these lanthanide chelates can be used in place of other labels typically used in ELISA-based procedures. Cumulatively, these factors contribute to the high sensitivity, low backgrounds, wide dynamic range, superior stability, and excellent flexibility characteristic of TRF [2,3].

In 1998, Berthet et al. identified a low molecular weight protein secreted by pathogenic M. tuberculosis, which was encoded by 1hp gene (303 bp) and belonged to ESAT6 family, named CFP-10 [4]. CFP-10 was secreted by M. tuberculosis and Mycobacterium bovis, lost in BCG and other non-pathogenic M. tuberculosis [5], and its coded gene was located at RD1 region [6]. It is feasible to detect CFP-10 for the early diagnosis of tuberculous pleural effusion, since CFP-10 is secreted at the early stage of M. tuberculosis life cycle [7]. Thus, detection of CFP-10 is proposed to shorten the diagnostic time of tuberculous pleural effusion, which has an obvious advantage over mycobacterial culture in clinical setting.

Here we reported the double-antibody sandwich-based TRF of CFP-10, which was tested in clinical specimens to differentiate tuberculous pleural effusion from malignant one, demonstrating that the novel assay may be used for the rapid diagnosis of tuberculous pleural effusion.

2. Materials and methods

2.1. Reagents

The monoclonal antibodies C01400M and C01398M against CFP-10 were purchased from Meridian Life Science (Memphis, TN, USA). For the double antibody sandwich-based assay, C01400M was the capturing antibody, and C01398M, the detecting antibody labeled with Eu^{3+}. CFP-10 reference sample was CFP10-SA fusion protein as generated before [8]. DELFIA Eu-Labelling reagent 1244–301 was purchased from PerkinElmer (Waltham, MA, USA), and Sephadex G-50, from Amersham Pharmacia Biotech (Piscataway, NJ, USA). CFP-10 quality control protein was purchased from Rekom Biotech in Granada, Spain. The five M. tuberculosis secretory proteins including ESAT6(Rv3875), Rv2801c, Rv1991, Rv1495 and 38KD(Rv0934) protein were gifted by Professor Xu-xia Zhang at Department of Bacteriology and Immunology, Beijing Chest Hospital. All other chemicals were of analytical reagent grade.

2.2. Instruments

The following instruments were used in the study: DR6608 time-resolved fluorescence analyzer (Foshan Daan, Guangzhou, China), DEM-3 auto plate washer (Beijing Tuopu Analytical Instrument, Beijing, China), and KJ-201C oscillator (Jiangsu Kangjian Medical Apparatus, Jiangsu, China).

2.3. Collection of clinical specimens

In total, 247 malignant pleural effusion specimens and 235 tuberculous pleural effusion specimens were collected by pleural puncture from the patients at Beijing Chest Hospital. Among the lung cancer patients, there were 179 male (aged 23–87), and 68 female (aged 24–88), while there were 147 male (aged 17–82) and 88 female (aged 17–82) among the patients with tuberculous pleural effusion, whose diagnosis were performed through measurement of ADA level, and further confirmed by mycobacterial culture and pleural biopsy, and further successful treatment. All the specimens were stored at −20 °C after centrifugation (8,000r/min, 15 min) and removal of the sediments. This study was approved by the Committee on the Ethics at Beijing Chest Hospital, and the methods in this study were received consent from each patient mentioned above.

2.4. Methods

The microplates coated with anti-CFP-10 antibody were prepared as follows: C01400M antibody was diluted into 3 μg/ml with carbonate buffer (50 mM, pH9.6), and then the resultant coating buffer (100 μl) was added into each well of the 96 microplates and incubated at 4 °C overnight. After the coating buffer was removed, the blocking buffer (150 μl) was added into each well. After incubation at room temperature for 2 h, the blocking buffer was removed and the microplates were stored at 4 °C.

The Eu^{3+}-labeled anti-CFP10 antibody was prepared as follows: 1 mg C01398M antibody was purified and concentrated by use of a centrifuge tube with 50kD cut-off filter membrane before labeling. The purified C01398M antibody was diluted in Eu^{3+} chelate-labeling buffer (0.1 mol/L sodium carbonate, pH9.3) to a final concentration of 1 g/L, and then 0.2 mg DELFIA Eu-N1 ITC chelate was added. The mixture was incubated at room temperature overnight. Free chelates were removed from the labeled antibody by use of gel filtration on a Sephadex G-50 column (1.5 cm × 40 cm). Finally, the Eu^{3+}-labeled anti-CFP10 antibody C01398M (100ug/L) was stored in the assay buffer (50 mmol/L Tris–HCl buffer, 0.5% BSA, 0.9% NaCl, 0.05% Tween-20 and 0.05% NaN3, pH 7.8) at 4 °C.

The CFP10-SA reference standard was diluted into 0, 0.3, 1.5, 7.5, 37.5 and 187.5 ng/ml with the dilution buffer (0.2% BSA, 0.1% NaN_3, and 50 mM Tris–HCl, pH7.8.), and stored at −20 °C

The measurement of CFP-10 by TRF method was done according to the two-step procedure. Briefly, 100 μl of CFP10-SA reference standards or specimens were added duplicately into the 96 microplate coated by C01400M antibody, and incubated at room temperature for 1 h. The microplate was then washed with the washing buffer (0.05 mmol/L PBS, pH 7.6, 0.01% Tween-20.) for 5 times. After washing, 100uL Eu^{3+}-labelled C01398M antibody was added into each well and incubated at room temperature for 1 h. The microplate was then washed for 6 times. Finally, 100 μl of DELFIA Dissociation-enhancement Solution was added into the microplate, and incubated at room temperature for 5 min. The measurement was carried out on a DR6608 time-resolved fluorescence analyzer with the excitation wavelength 340 nm, the emission wavelength 613 nm, 1 s measuring time, 400μs delay time, and 400μs counting time.

2.5. Method evaluation

Three batches of TRF reagents were stored at 4 °C for 1.5 years, or 37 °C for 7 days, and further measured respectively to test their stability.

To validate the feasibility of the novel TRF method in quantitating CFP-10, CFP-10 quality control protein with the following concentrations of 12, 50 and 160 ng/ml were prepared. Eight repetitions were set for each concentration, and the three batches of TRF reagents with different storage times and conditions mentioned above were used for evaluation. The mean value, standard deviation and coefficient of variation (CV) were calculated accordingly.

CFP10-SA fusion protein was chosen as the reference standard to determine the detection limit of the novel TRF method [8]. In brief, CFP10-SA and SA-CFP10 fusion proteins were expressed as