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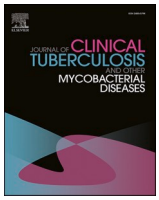

Case Report

Diagnostic value of tuberculosis-Specific antigens Ag85B, ESAT-6 and CFP10 in pulmonary tuberculosis

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ABSTRACT

Objectives: To assess the diagnostic value of tuberculosis-secreted Ag85B, ESAT-6 and CFP10 in pulmonary tuberculosis.
Method: Immunohistochemical (IHC) and acid-fast staining were performed, the detection efficiency of the two methods were analysed with chi-square test.
Results: Acid-fast staining demonstrated a sensitivity of (18/69, 26.09%) and a specificity of 100%. IHC staining for Ag85B, ESAT-6 and CFP10 was positive in all 18 cases with positive acid-fast staining and additionally positive in 25,36 and 30 cases, respectively, that were acid-fast-negative. The sensitivities of IHC staining for Ag85B, ESAT-6 and CFP10 were 62.32%, 78.26% and69.57%, respectively, while their specificities were 86.57%, 85.07% and 91.04%, respectively. Joint antigen analysis revealed that simultaneous positivity for two or three of Ag85B, ESAT-6 and CFP10 increased specificity (95.52% to 100%) but decreased sensitivity (49.28% to 62.31%). The presence of any one of the three antigens elevated sensitivity (81.16% to 88.41%) but lowered specificity (68.66% to 77.61%). Specificity reached 100% for concurrent positivity of all three antigens, while a sensitivity of 88.41% was observed for positivity of any one antigen.
Conclusions: Ag85B, ESAT-6, and CFP10 exhibits greater sensitivity in pulmonary tuberculosis and combined antigen testing enhances the diagnostic accuracy.

1. Introduction

Tuberculosis, caused by Mycobacterium tuberculosis (MTB) infection, was identified in the 2022 WHO Global Tuberculosis Report as the second-leading cause of death from infectious diseases worldwide. This chronic infectious disease can affect various organs except teeth, hair and nails, with lung being the most frequently involved organs, accounting for approximately 80 % of cases [1,2]. Early diagnosis and timely treatment of lung TB are critical to ending the transmission of TB. Pathological examination remains the gold standard for diagnosing lung tuberculosis. Acid-fast staining is in common use but suffer from limited sensitivity and specificity [3]. MTB culture requires stringent laboratory, which hinder its widespread adoption in primary care settings. In addition, the long culture duration makes it difficult to meet the need for the early diagnosis of tuberculosis. Molecular biology techniques have greatly improved the detection rates. Advent of Xpert MTB/RIF (Xpert), an automated molecular detection system that simultaneously detects TB and resistance to rifampicin, proved to be a breakthrough in TB diagnosis. However, the difficulty associated with specimen acquisition in paucibacillary cases persists [4]. Antigen detection offers a promising diagnostic avenue, especially with advances in understanding the immune mechanisms of tuberculosis. Among various MTB antigens, secretory antigens are crucial for diagnosis. Ag85B, a major secretory antigen of MTB, accounts for 5–6 % of the total secreted proteins and is highly expressed [5]. ESAT-6 and CFP10 are primary components of MTB's early secretory proteins, forming a strong 1:1 complex, exclusively present in pathogenic MTB and absent in most non-tuberculosis mycobacteria and bovine BCG, thus conferring high specificity. Although some studies have detected these antigens in tuberculous lesions, research remains limited [5,8,11,17]. The 2017 Chinese Expert Consensus on Pathological Diagnosis of Tuberculosis acknowledges the potential of immunohistochemical staining for detecting MTB-specific antigens in lesions, but emphasizes the need for more clinical translational studies due to the absence of specific antibodies and unified

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interpretation standards for clinical diagnosis [8]. This study aims to investigate the expression of MTB-specific antigens Ag85B, ESAT-6, and CFP10 in lung tissue using IHC and evaluate their diagnostic utility.

2. Materials and methods

2.1. Experimental subjects

136 lung tissue specimens surgically removed and paraffin-embedded at the Pathology Department, the Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan Province, China from April 2019 to October 2022 were retrospectively selected. These were divided into two groups: pulmonary tuberculosis group (69 cases) and non-tuberculosis (67 cases). The pulmonary tuberculosis group's pathology conformed to Category I or II of the 2017 Chinese Expert Consensus on Pathological Diagnosis of Tuberculosis (Song and Che, 2018). Class I criteria involved definitive tuberculosis diagnosis with corresponding pathological tissue and cell changes, along with etiological evidence. Class II criteria included suggestive diagnoses with tuberculosis-like pathological changes but without clear etiological evidence. All patients in the tuberculosis group showed clinical evidence supporting the pulmonary tuberculosis diagnosis and responded to anti-tuberculosis treatment. The non-tuberculosis group comprised 33 lung cancer cases and 34 normal lung tissues (lung resection margins), with no history of tuberculosis and negative IGRAs in all cases.

2.2. Experimental methods

Each paraffin block was sectioned continuously at a thickness of 4 μm to prepare five sections: one for HE staining, one for acid-fast staining, and three for IHC. Pathologists reviewed the HE-stained slides for diagnostic accuracy before performing acid-fast and IHC staining.

2.3. Acid-fast staining (Ziehl-Neelsen method)

The process followed the manufacturer's protocol (AFB Stain Kit, Baso, Zhuhai, China), Involved fixation, dewaxing, and washing of paraffin sections, followed by staining with carbolic acid red solution for 10–15 min. The sections were then decolorized with acidic alcohol until the red color vanished, counterstained with methylene blue for 20–30 s, washed, and sealed with neutral gum after air-drying.

2.4. Immunohistochemical staining

Staining was conducted using the SP (streptavidin peroxidase) method, with primary antibodies diluted as follows: Ag85B (bs-9268R, Beijing Boersen Ancient Biotechnology Co., Ltd.1:300 dilution), ESAT-6 (bs-13107R, Beijing Boersen Ancient Biotechnology Co., Ltd.1:300 dilution), CFP10(ABIN285580, Antibodies-online, Philadelphia, PA, USA, 1:400 dilution). Microwave antigen retrieval preceded the staining, performed according to the reagent kit instructions. DAB (dimethylobenzidine) was employed for chromogenic staining, hematoxylin for counterstaining, and PBS (phase buffer saline) as a negative control. All slides were independently reviewed by two senior pathologists. In the case of discordant results, a third pathologist arbitrated, and the majority opinion was accepted.

2.5. Statistical analysis

The SPSS 27.0 software facilitated the data analysis, with P-values less than 0.05 denoting statistical significance. Qualitative data were presented as frequencies (%), and the chi-square test was used for inter-group comparisons.

3. Results

3.1. Patient demographics and clinical characteristics

Table 1 outlines the demographic and clinical attributes of the participants. The study analyzed 136 lung tissues, including 69 from patients with pulmonary tuberculosis and 67 from non-tuberculosis. In the tuberculosis group, there were 45 males (65 %) and 24 females (35 %), with an average age of 51.75 ± 10.27. The non-tuberculosis group comprised 35 males (52 %) and 32 females (48 %), with an average age of 50.91 ± 13.82. All cases have lung CT findings indicating lung lesions, most (80 %, 55/69) presented with cough, a few (20 %, 14/69) were asymptomatic, some patients exhibited symptoms as fever, night sweats, or weight loss.

3.2. Acid-fast staining results

Under oil immersion mircroscopy, acid-fast bacilli appear red, predominantly rod-shaped and slightly curved, occasionally forming longer spiral structures (Fig. 1A). They are frequently located in the central areas of necrosis or at the interface of necrosis and epithelioid granulomas. Of the tuberculosis cohort, 18 of 69 cases (26.09 %) tested positive for acid-fast bacilli, while all 67 control specimens were negative. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of acid-fast staining in this study were calculated as 26.09 %, 100.0 %, 100.0 % and 56.78 %, respectively (Table 2).

3.3. Immunohistochemical staining results

Initially, samples positive for acid-fast bacilli were selected for IHC staining to assess the distribution of IHC positive signals. The findings revealed that Ag85B, ESAT-6, and CFP10 all exhibited dark brown granules, primarily localized in necrotic tuberculosis tissue and adjacent macrophages and Langhans' giant cells. The positive signal characteristics included: 1) clustering, small patches, or snowflake-like staining; 2) darker central coloring fading to lighter at the periphery; 3) predominantly dotted distribution; 4) frequent expression in and around necrotic tissue. The intensity of these signals correlated with the number of acid-fast bacilli, and their expression range was broader, mirroring the distribution of these bacilli **(**Fig. 1**C- E).** The non-tuberculosis control immunohistochemical staining exhibited no specific pattern of distribution and presented minimal light brown signals **(**Fig. 1**F).**

3.4. Comparative analysis of two detection schemes

The comparative analysis of acid-fast staining and IHC staining indicated that IHC staining for Ag85B, ESAT-6 and CFP10 was positive in all 18 cases with positive acid-fast staining and additionally positive in 25,36 and 30 cases, respectively, that were acid-fast-negative. IHC

Table 1
Demographic and clinical characteristics of patients.

Characteristics	TB N=69(%)	Non-TB N=67(%)
Gender		
Male	45(65)	35(52)
Female	24(35)	32(48)
Age(average)	51.75 ± 10.27	50.91 ± 13.82
Symptoms		
cough	55(80)	32(48)
Fever	25(36)	5(7)
Night sweat	17(25)	3(4)
Weight loss	10(14)	0
No symptoms	14(20)	46(69)
lung lesions	69(100)	67(100)

Abbreviations: TB, tuberculosis; Non-TB, non-tuberculous.

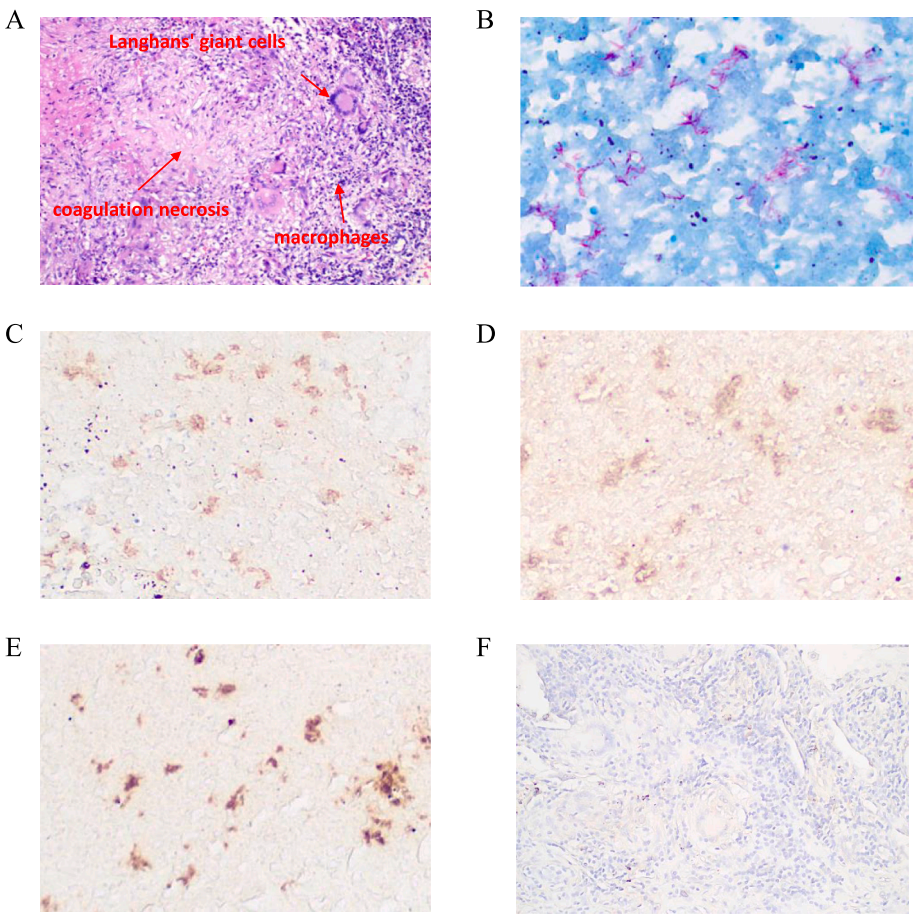

Fig. 1. HE, Acid-Fast and Immunohistochemical Staining of Pulmonary Specimens. Note: Figures A-E depict continuous sections of the same block. A shows HE staining of pulmonary Tuberculosis tissue, highlighting coagulation necrosis with granulomatous inflammation, including macrophages and Langhans' giant cells (Arrow pointing) (×200); B presents acid-fast staining, with red, rod-shaped, slender, and slightly curved bacilli (oil immersion mircroscopy); C-E exhibit immunohistochemical staining for Ag85B, ESAT-6, and CFP10, respectively, showing diffuse typical brown particles in necrotic lesions, consistent with acid-fast bacilli distribution, but more extensive (SP, ×200). F serves as the negative control.

Table 2
Comparison of Detection Efficiency Among Two Schemes and diagnostic value.

Diagnostics			TB	Non-TB	Sensitivity	specificity	PPV	NPV
			$n = 69$	$n = 67$	(%)	(%)	(%)	(%)
AFB		+	18	0	26.09	100	100	56.78
		−	51	67				
IHC	Ag85B	+	43	9	62.32	86.57	82.69	69.05
		−	26	58				
	ESAT-6	+	54	10	78.26	85.07	84.38	79.17
		−	15	57				
	CFP10	+	48	6	69.57	91.04	88.89	74.39
		−	21	61				

Abbreviations: TB, tuberculosis; Non-TB, non-tuberculous; PPV, positive predictive value; NPV, negative predictive value; AFB, acid-fast bacilli, IHC, immunohistochemical staining;Ag85, antigen 85; ESAT-6,early secretory antigenic target-6;CFP-10,culture filtrate protein-10.

was more sensitive than acid-fast staining (26.09 %). Among the three antigens, ESAT-6 showed the highest sensitivity (78.26 %), followed by CFP10 (69.57 %) and Ag85B (62.32 %). The specificity of acid-fast staining was 100 %, while the specificities of the IHC for the three antigens were between 85.07 % and 91.04 % (Table 2).

3.5. Antigen joint analysis

3.5.1. Simultaneous positivity of two or three antigens

Joint detection of Ag85B, ESAT-6, CFP10, when they are positive for both or all of the three antigens, yielded the following sensitivities and specificities for the groups: ESAT-6 + CFP10 (sensitivity: 62.31 %, specificity: 98.51 %), Ag85B + CFP10 (sensitivity: 50.72 %, specificity: 100 %), Ag85B + ESAT-6 (sensitivity: 57.97 %, specificity: 95.52 %), and Ag85B + ESAT-6 + CFP10 (sensitivity: 49.28 %, specificity: 100 %) (Table 3).

3.5.2. Positive for anyone of two or three antigens

When considering positivity for anyone of two or three antigens (Ag85B, ESAT-6, and CFP10) as a positive result, the sensitivities and specificities were as follows: ESAT-6/CFP10 (sensitivity: 85.51 %, specificity:77.61 %), Ag85B/CFP10(sensitivity: 81.16 %, specificity:

Table 3
Analysis of diagnostic value of antigen combination.

		TB	Non-TB	Sensitivity	specificity	PPV
		n = 69	n = 67	(%)	(%)	(%)
ESAT-6 + CFP10	+	43	1	62.31	98.51	97.73
	−	26	66			
Ag85B + CFP10	+	35	0	50.72	100	100
	−	34	67			
Ag85B + ESAT-6	+	40	3	57.97	95.52	93.02
	−	29	64			
Ag85B + ESAT-6 + CFP10	+	34	0	49.28	100	100
	−	35	67			

Abbreviations: TB, tuberculosis; Non-TB, non-tuberculous; PPV, positive predictive value; Ag85, antigen 85; ESAT-6, early secretory antigenic target-6; CFP-10, culture filtrate protein-10.

77.61 %), Ag85B/ESAT-6 (sensitivity:82.61 %, specificity:76.12 %), and Ag85B/ESAT-6/CFP10(sensitivity:88.41 %, specificity: 68.66 %) (Table 4).

4. Discussion

The fundamental pathological alterations in tuberculosis encompass inflammatory exudation, proliferation, and caseous necrosis. These changes are influenced by factors such as the virulence of MTB, bacterial load, allergic reactions, and the body's innate resistance [6,14,15]. Gross examination typically reveals tuberculosis specimens as grayish-yellow, finely textured, and cheese-like necrotic tissue (caseous necrosis) [6], while its microscopic hallmark is granuloma with caseous necrosis. It is imperative to recognize that, despite their specificity, these gross, histological, and cytological features of tuberculosis can also manifest in other granulomatous diseases. Consequently, a definitive diagnosis of tuberculosis requires corroborating pathogen-specific evidence from additional tests.

Acid-fast staining is a prevalent clinical method for detecting MTB, noted for its simplicity and cost-effectiveness. However, it has limitations, including a low positivity rate and potential for misdiagnosis. The sensitivity of acid-fast staining in this study (26.09 %) is consistent with the results reported in the literature [7,9,13]. It is crucial to acknowledge that acid-fast staining results for Mycobacterium leprae, non-tuberculous mycobacteria, some Legionella, and Nocardia can also be positive, necessitating further molecular pathological testing for accurate differentiation [12,16].

Beyond bacteriological assessments, specific antigens are crucial for pathogen diagnosis. The application of IHC to detect MTB-specific antigens in tissue samples has garnered increasing attention [6]. This study utilized IHC to identify Ag85B, ESAT-6, and CFP10 in pulmonary tuberculosis tissue. The positive antigen signals were predominantly found in and around necrotic tuberculosis lesions, correlating with the distribution of acid-fast bacilli and exhibiting a more extensive range.

Table 4
Diagnostic value analysis of any positive antigen combination.

		TB	Non-TB	Sensitivity	specificity	NPV
		n = 69	n = 67	(100 %)	(100 %)	(100 %)
ESAT-6/CFP10	+	59	15	85.51	77.61	83.87
	−	10	52			
Ag85B/CFP10	+	56	15	81.16	77.61	80.00
	−	13	52			
Ag85B/ESAT-6	+	57	16	82.61	76.12	80.95
	−	12	51			
Ag85B/ESAT-6 /CFP10	+	61	21	88.41	68.66	85.19
	−	8	46			

Abbreviations: TB, tuberculosis; Non-TB, non-tuberculous; NPV, negative predictive value; Ag85, antigen 85; ESAT-6, early secretory antigenic target-6; CFP-10, culture filtrate protein-10.

This finding aligns with previous research, reflecting the nature of secreted antigens being present not only within cells but also in the surrounding environment [8,10,11,17]. The sensitivity of IHC staining for Ag85B, ESAT-6, and CFP10 was 62.32 %, 78.26 %, and 69.57 %, respectively, with corresponding specificities of 86.57 %, 85.07 %, and 91.04 %. Early studies applying IHC to detect Ag85B expression in diseased tissues, such as the lungs, kidneys, and lymph nodes, have provided valuable insights into tuberculosis pathology, the sensitivity ranged from 50.50 % to 58.70 % and the specificity was 100 % [5]. The sensitivity of IHC in detecting ESAT-6 in renal tuberculosis tissue, as reported by Zhao N [18], was 100 %, with specificity ranging from 87.10 % to 92.00 %. Research on CFP10 is limited, with some studies conducted in animal models [3,14,15]. The varied sensitivity and specificity observed in different studies could be attributed to factors such as diverse diseased tissues, antibody types and sources, and most importantly, the lack of a unified standard for interpreting IHC staining results. This variability is especially notable in the context of weak positive signals, which are challenging to judge accurately. It is suggested that both signal intensity and distribution should be incorporated into interpretation criteria, potentially enhancing clinical relevance. However, this necessitates further large-scale studies and validation by pathologists and clinical experts.

This study discovered that the intensity of immunohistochemical signals for Ag85B, ESAT-6, and CFP10 correlates with the quantity of acid-fast bacilli, and the range of signal expression aligns with the distribution of these bacilli, tending to be more extensive. This could be because these antigens are not only present within MTB cells but are also secreted externally and subsequently phagocytized by macrophages and multinucleated giant cells. Hence, IHC detection of these antigens can improve the sensitivity of tuberculosis diagnosis. This study and previous researches suggest that, for the pathological diagnosis of tuberculosis, IHC exhibits higher sensitivity than acid-fast staining [5,8,10,17,18]. In this study, the comparative analysis of the two methods also indicated that IHC staining for Ag85B, ESAT-6 and CFP10 was positive in all cases with positive acid-fast staining and additionally positive in part of other case that were acid-fast-negative. Thus, IHC holds supplementary diagnostic value in tuberculosis cases where acid staining negative. Moreover, IHC is operationally straightforward, with easily observable positive signals and modest laboratory requirements, enhancing its feasibility.

Past studies and the current findings indicate that no single antigen achieves ideal sensitivity and specificity for tuberculosis diagnosis. Hence, the combined detection of multiple antigens is often employed. This study demonstrated that when three antigens are simultaneously positive, both specificity and positive predictive value are 100 %, which is highly significant for the confirming diagnosis of tuberculosis. The sensitivity of detecting any one of the three antigens is 88.41 %, and the negative predictive value is 85.19 %. Consequently, negativity for all three antigens bears considerable significance in clinically excluding tuberculosis.

Limitations of this study include its retrospective nature and reliance on paraffin-embedded lung tissue, precluding MTB culture and lacking a definitive gold standard for MTB diagnosis. The sample size is also relatively small. Future studies, preferably multi-center and large-scale prospective ones, are warranted to further substantiate these findings.

5. Conclusion

IHC staining for Ag85B, ESAT-6, and CFP10 exhibits greater sensitivity compared to acid-fast staining in paraffin-embedded lung tissue and provides valuable diagnostic support, particularly in cases where acid-fast staining are negative.

Combined antigen testing enhances the diagnostic accuracy for pulmonary tuberculosis, with simultaneous positivity of all three antigens being crucial for confirming tuberculosis, while their collective negativity is significant in ruling out the disease.

Ethical approval statement

The present study was approved by the Medical Ethics Committee of North Sichuan Medical College (approval no. 2022ER296-1). All patients provided written informed for participation in the present study.

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CRediT authorship contribution statement

Jie Sun: Data curation, Formal analysis, Funding acquisition, Writing – original draft. **Xinchun Zhou:** Investigation, Methodology. **Jiang Yu:** Methodology. **Shiyu Fang:** Methodology. **Shaoqi Duan:** Investigation, Methodology. **Fengjun Liu:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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