The Performance of Real-Time Polymerase Chain Reaction in Patients with Scanty Positive Acid-Fast Bacilli Sputum Smear in Diagnosis of Pulmonary Tuberculosis: 5-Year Retrospective Study

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ABSTRACT

Objective: To assess the performance of real-time polymerase chain reaction (RT-PCR) to diagnosis pulmonary tuberculosis in patients with scanty positive acid-fast bacilli sputum smears, in a single hospital.

Materials and Methods: All patients, who had scanty positive AFB sputum smears in Songklanagarind Hospital; between 2015 and 2019 were included. Demographic data, clinical data, radiographic findings, RT-PCR and mycobacterial culture results were reviewed.

Results: From a total of 269 patients reporting scanty AFB smears, 116 patients (43.1%) had cultures confirmed as *M. tuberculosis*. From overall, samples from 92 patients with scanty AFB smear were processed for RT-PCR. There were 26 (28.3%) isolates having positive RT-PCR test results. Of these 26 isolates that RT-PCR positive, 25 (96.2%) were culture positive, while only 1 (3.8%) were culture negative. A remaining 66 samples that RT-PCR negative, 15 (22.7%) were culture positive for tuberculosis. Using mycobacterial cultures as the gold standard, the sensitivity, specificity, positive predictive value and negative predictive value of RT-PCR were 62.5%, 98.1%, 96.2%, and 77.3%, respectively. Pulmonary consolidation and cavity on chest radiograph were associated with the growth of *M. tuberculosis*, with an OR of 2.3 (95% C.I. 0.26-0.73) and 3.4 (95% C.I. 1.2-9.9), respectively.

Conclusion: Less than half of the patients with scanty smears had culture-confirmed tuberculosis; RT-PCR also has low sensitivity. Consequently, a negative RT-PCR does not exclude tuberculosis; especially in cases of a high index for clinical suspicion. Radiographic findings; including pulmonary consolidation and cavities, are helpful predictors for supporting this diagnosis.

Keywords: Performance; scanty acid fast bacilli; pulmonary tuberculosis; polymerase chain reaction (Siriraj Med J 2021; 73: 445-450)

INTRODUCTION

Pulmonary tuberculosis remains a serious, worldwide public health problem.[1] Thailand remains on the list of the world's 14th highest burden country of tuberculosis.[2-3] The high number of tuberculosis cases and deaths indicates that actions are urgently needed to reduce tuberculosis incidence. Rapid identification and treatment of new cases is the keystone of tuberculosis control.[4-5] Acid Fast Bacilli (AFB) sputum smear microscopy is widely used as the diagnostic test for mycobacterial disease; as it is a simple, rapid and cost-effective method for diagnosing tuberculosis.[6]

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Ziehl-Neelsen (ZN) stained smear is a conventional method; however, auramine-staining has reported to have 10 percent more sensitivity with similar specificity.[7-12] Since 2006, the central laboratory of Songklanagarind Hospital has applied the auramine fluorescence microscopic technique as its screening test for all requested sputum acid fast smears, before being confirmed with ZN-stained. With this more sensitive staining technique, we found increasing reports of scanty AFB in sputum smears.

The scanty AFB results were defined as: the presence of less than 10 bacilli in the 100 oil-field on microscopy. The International Union Against Tuberculosis and Lung Disease (IUATLD), along with the World Health Organization (WHO), previously recommended that additional sputum AFB smears should be repeated in this degree of positivity, when reported as doubtful.[13-14] In 2013, WHO revised the case definitions of pulmonary tuberculosis.[15] Cases with scanty AFB on microscopy will be considered as sputum smear positive tuberculosis. This lowering diagnostic threshold thereby increases case detection rates and treatment initiation. However, this degree of positive acid fast smear might not well correlate with cultures, as it could reflect non-tuberculous mycobacteria, or contamination by environmental mycobacteria.[16-17] The nucleic acid amplification test (NAAT) is useful as it can rapidly detect *M. tuberculosis* bacteria in specimens within hours, while the turnaround time of mycobacterial cultures requires 2 to 6 weeks for reporting. However, the NAAT performance to detect *M. tuberculosis* in scanty positive AFB sputum smears remains a challenge, because of low bacillary loads.[20-22] Hence, the aim of this study was to explore the yield of scanty positive acid fast sputum smears, and the multiplex Real Time-Polymerase Chain Reaction (RT-PCR) Anyplex™ II MTB/MDR Detection technique to diagnose pulmonary tuberculosis.

Objectives

The purposes of this study were:

1. To determine the performance of real-time polymerase chain reaction in patients with scanty positive acid-fast bacilli sputum smear in diagnosis of pulmonary tuberculosis

2. To explore the factors that can predict the growth of *M. tuberculosis* in patients with scanty positive AFB sputum smears

MATERIALS AND METHODS

Study design and population

A retrospective review of medical and microbiological records of all suspected respiratory tuberculosis patients, whose sputum were sent for AFB staining, from January, 2015 to December, 2019 in Songklanagarind Hospital, an 800-bed, teaching-based, tertiary care hospital in Songkhla province, Southern Thailand. The investigational protocol was approved by the Institutional Review Boards of Faculty of Medicine, Prince of Songkla University: REC. 63-287-14-1. Enrollment criteria of the patients were: (1) aged > 15 years, (2) had at least one specimen showing a scanty positive acid fast smear, (3) having at least one sputum specimen sent for mycobacterial culture. Patients were excluded if they: (1) had recent or ongoing treatment with anti-tuberculous agents, (2) had no obtained mycobacterial culture, (3) had not undergone chest radiograph during sputum collection.

Data collection

The patient's demographic data, presenting symptoms, underlying medical illness (es), results of mycobacterial culture and multiplex RT-PCR, chest radiographic findings, rate of anti-tuberculosis treatment and adverse drug events were all recorded.

As the laboratory routine works, sputum specimens are processed and performed for decontamination and concentration via a standard method, preparation of slides and fluorochrome staining with auramine O. With auramine O staining, mycobacteria appear as bright, yellow fluorescent rods under a fluorescent microscopy. Slide with a presence of fluorescent rods are ZN stained, and evaluated by using a conventional light microscope. Performing a ZN stain, after initial auramine O staining, is accepted as standard practice in Songklanagarind Hospital. The number of acid-fast bacilli observed has been quantified according to the IUALD and the WHO scales. Mycobacterial cultures are the gold standard for bacteriological confirmed diagnosis of tuberculosis, and growing bacteria is required to perform drug-susceptibility testing. Sputum specimens were cultured in liquid (7H9 broth) medium, with use of the automated Mycobacterial Growth Indicator Tube system and solid Löewenstein-Jensen medium.

Eligible patients were patients who had scanty positive acid fast bacilli of sputum smear, which was defined as: having presence of at least one of two, or three slides reported to have less than 10 acid fast bacilli (AFB) found in 100 oil fields; according to the WHO scale. Regarding the results of sputum mycobacterial cultures, they were assigned as either a positive culture for *M. tuberculosis* (positive C/S MTB) or negative culture for *M. tuberculosis* (negative C/S MTB). Negative C/S for MTB was defined by there being at least one or more sputum specimens sent for mycobacterial cultures

reported as growing non-tuberculous mycobacterial (NTM), or no mycobacterial growth.

As part of a diagnostic investigation, direct molecular testing was performed on 92 scanty sputum smears, submitted by physician request using the Anyplex™ II MTB/MDR Detection kit. This assay is a semi-automated system, and provides rapid results within 3-4 hours of sample receipt. This technique also has a lower rate of error and contamination [11], compared to the Line probe assay. Before performing the multiplex RT-PCR technique, DNA of *M. tuberculosis* was extract by using DNA-extraction solution, provided in the kits. Anyplex™ II MTB/MDR Real-time Detection was performed following the directions provided by the manufacturer. Amplification and detection were performed on a Rotor-Gene 3000 instrument, for all sample extracts. *M. tuberculosis* detection targeted the IS6110 and MPB64 genes. Result interpretation was performed automatically, using the instrument's software according to threshold and cutoff values outlined by the manufacturer (16). Overall, the Anyplex MTB/NTM assay demonstrated sensitivity, specificity, PPV, and NPV of 86%, 99% 96%, and 95%, for *M. tuberculosis* detection compared with mycobacterial cultures.

Statistical analysis

To describe the variables characteristics, these were expressed as mean with standard deviation, median with range, proportion in percentage and ratio. To examine differences between 2 groups of variables these were analyzed by Fisher's exact or χ^2 test. The categorical variables were analyzed by Student's t-test or Mann-Whitney U test for the continuous variables, according to types of its distribution. Statistical analysis was performed with SPSS software version 23 (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered statistically significant.

RESULTS

During the five-year study period, 416 patients met criteria for inclusion in the study. From this, 147 patients (35.3%) were excluded for the following reasons: 126 patients had recent or ongoing treatment with anti-tuberculous agents, 2 patients underwent no chest radiograph during sputum collection, and 19 had no obtained mycobacterial culture. In total, 269 patients were enrolled in this study (Fig 1).

Of the 269 patients with scanty positive AFB smears, 116 patients (43.1%) had positive culture for *M. tuberculosis*, while the remaining 153 patients (56.9%) had negative

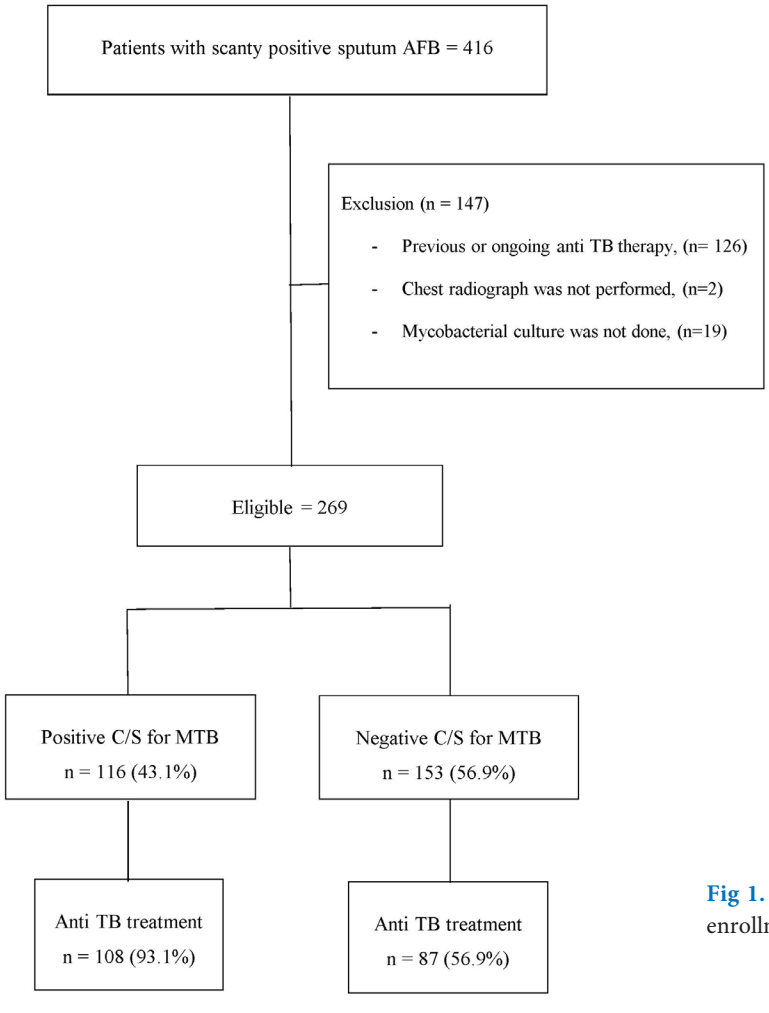

Fig 1. Flow diagram for patient enrollment.

cultures. There were no statistically significant differences between these 2 groups; in terms of: patient characteristics, presenting symptoms or comorbidities, as shown in Table 1. Means age of positive and negative mycobacterial culture groups were 55.6±18.7 years vs. 61.7±17.1 years, respectively (p=0.134). About two-thirds of patients in both groups were male. Pulmonary consolidation and cavity on chest radiograph were frequently detected in the culture positive group (44.8% vs. 26.1%, 10.3% vs. 3.2%, respectively, P<0.05), while normal chest radiograph favored the culture negative group (1.7% vs. 24.8%, P<0.05). Reticular lesions were commonly found in active respiratory tuberculosis; however, there was no statistically significant difference between positive and negative mycobacterial culture groups (P=0.676). From this, 93.1% of patients in the culture positive group received anti-tuberculosis treatment, compared to 56.9% of patients in the culture negative group (P<0.05). The percentage of adverse drug events were 26.7% in the positive culture group and 9.8% in the negative culture group (P =0.135)

There were 92 sputum scanty AFB smears sent for multiplex RT-PCR, and overall there were 26 (28.3%) cases having positive RT-PCR test results. Of these, 25 (96.2%) were culture positive, while only 1 (3.8%) were culture negative. A remaining 66 samples that RT-PCR negative, 15 (22.7%) were culture positive for tuberculosis. Using mycobacterial cultures as the gold standard, the sensitivity, specificity, positive predictive value and negative predictive value of RT-PCR were 62.5%, 98.1%, 96.2%, and 77.3%, respectively (Table 2). Overall, the diagnostic yield of multiplex RT-PCR in scanty AFB was relatively low. However, anti-tuberculous treatment should be initiated without delay in patients with positive RT-PCR, as it has a low, false positivity (3.8%).

DISCUSSION

Scanty positive AFB reporting accounted for one-third from all sputum smear positives in our institute. Of these, 41.3% grew *M. tuberculosis*. Similarly, previous studies showed that fewer than half of the smears, with scanty AFB, yielded positive cultures.[23-25] The chance of scanty positive AFB to be a positive culture for *M. tuberculosis* is limited, and might be due to low bacillary loads. False positive scanty sputum smears are possibly from non-tuberculous mycobacteria[26]; particularly asymptomatic patients without chest radiological evidence suggesting active tuberculosis.

As for the results of this study; 195 patients (72.5%) who had scanty positive AFB smears were being treated with anti-tuberculosis treatment. Interestingly, 12 out of 38 patients (31.6%), who had scanty AFB smears and negative mycobacterial cultures, were receiving anti-tuberculosis treatment; despite normal chest radiograph.

Our study also revealed that RT-PCR had low sensitivity for patients who had scanty positive AFB smears. The results are supported by IUATLD/WHO recommendations, in that a single negative NAA test could not exclude the diagnosis of pulmonary tuberculosis; especially in cases of moderate to high index of clinical suspicion. Chest radiographic findings are helpful in terms of prediction the following growth of *M. tuberculosis*. Chest radiographic findings indicate active respiratory tuberculosis; including, consolidation opacities, and cavitation, which are related to the growth of *M. tuberculosis*. Only 2 of our 116 patients (1.7%) having normal chest radiographic findings had *M. tuberculosis* in their sputum. Our findings suggest empiric anti-tuberculous treatment for all scanty positive AFB patients with radiographic findings suggestive of active tuberculosis, as this might be appropriate; whereas, the follow up approach is considered in patients who have normal chest radiography. This strategy is to avoid overtreatment and drug related complications.

There are some limitations in this study. First, tuberculosis diagnosis is based entirely on the detection of *M. tuberculosis* via culture techniques. Failure to isolate *M. tuberculosis* cannot definitely exclude a diagnosis of active tuberculosis. Second, our retrospective study may have introduced a selection bias, because approximately two-thirds half of the patients with scanty positive AFB smears did not performed RT-PCR tests. Thus, the outcome might not reflect the true performance of RT-PCR in all patients with scanty positive results.

CONCLUSION

Presence of scanty positive AFB in sputum smear was common, while only 43.1% of them were finally confirmed as tuberculosis by mycobacterial culture. Also, the RT-PCR sensitivity in scanty acid fast sputum smears is very low. Hence, if clinical suspicion is high, tuberculosis should not be ruled out based solely on negative RT-PCR results. Chest radiographic findings are helpful in determining empirical anti-tuberculosis treatment.

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TABLE 1. Demographic, clinical manifestations and laboratory results of patients with scanty AFB, and results of their mycobacterial culture.

Characteristics	Patients with scanty positive AFB (n=269)		
	Positive C/S for MTB n=116 (43.1%)	Negative C/S for MTB n=153 (56.9%)	*p*-value
Age in years (mean ±S.D.)	55.64 ±18.68	61.66 ±17.09	0.134
Male gender (%)	65.5%	57.5%	0.183
Presenting symptoms*			
Cough	63 (54.31%)	66 (43.14%)	0.069
Fever	22 (18.97%)	20 (13.07%)	0.187
Weight loss	20 (17.24%)	26 (16.99%)	0.957
Hemoptysis	8 (6.89%)	12 (7.84%)	0.769
Pleurisy	3 (2.58%)	2 (1.30%)	0.442
Dyspnea	13 (11.2%)	15 (9.8%)	0.709
Underlying diseases*			
HIV/AIDS	6 (5.17%)	9 (5.88%)	0.802
Diabetes	14 (12.07%)	23 (15.03%)	0.485
Malignancy	23 (19.83%)	43 (28.1%)	0.118
Renal failure	3 (2.59%)	5 (3.27%)	0.744
Immuno-suppressive therapy	6 (5.17%)	7 (4.58%)	0.821
Chest radiograph finding*			
Normal	2 (1.72%)	38 (24.84%)	<0.05
Consolidation	52 (44.82%)	40 (26.14%)	<0.05
Reticular	22 (18.97%)	26 (16.99%)	0.676
Cavity	12 (10.34%)	5 (3.27%)	0.018
Miliary	6 (5.17%)	1 (0.65%)	0.021
Pleural effusion	15 (12.93%)	12 (7.84%)	0.169
Nodule/mass	28 (24.14%)	51 (33.33%)	0.101
Received Anti-TB treatment	108 (93.1%)	87 (56.86%)	<0.05

Plus–minus values are means ±SD

Abbreviations: AFB=acid fast bacilli, C/S= Culture, MTB= *Mycobacterium tuberculosis*, HIV/AIDS = Human immunodeficiency virus/ Acquired Immune Deficiency Syndrome

*Patients may have had >1 manifestation.

TABLE 2. The results of multiplex real-time polymerase chain reaction of patients with scanty positive AFB sputum smears and mycobacterial culture.

	Positive C/S for MTB (n=40)	Negative C/S for MTB (n =52)	Total (n=92)
Positive PCR for MTB (n= 26)	25	1	26
Negative PCR for MTB (n= 66)	15	51	66
	40	52	92

Abbreviations: AFB=acid fast bacilli, PCR = Polymerase Chain Reaction, C/S = Culture, MTB=*Mycobacterium tuberculosis*, scanty AFB= positive acid fast staining but < 10 AFB/100 OF

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