



Identification and Isolation of *Mycobacterium tuberculosis* from Iranian Patients with Recurrent TB using Different Staining Methods

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ABSTRACT

Re-activation of tuberculosis (TB) and the emergence of multi-drug resistance (MDR) isolates is a serious problem in the worldwide. Thus, accelerating the recognition of resistance of *Mycobacterium tuberculosis* isolated from TB patients is important. The aim of this study was to identifying and isolation of *M. tuberculosis* isolates from Iranian patients with recurrent TB using different staining methods. During 16 months from October 2013 to February 2015, 176 sputum samples were collected from patients suspected with TB who were under treatment or had the infection with TB resistant to treatment referred to the Health Centre in Sari and Ghaemshahr. The sputum specimen's smears provided with four specific staining techniques. Data analysis was performed using t-test and descriptive statistics frequency distribution by SPSS-version 16 software. And P-values <0.05 was considered statistically significant. Of total 176 tuberculosis patients, 28 cases (15.9%) were AFB smear-positive sputum and also 17 cases (9.65%), 11 (6.25%) were male and female, respectively. The mean age of patients was 47±2 years. The sensitivity of Ziehl-Neelsen stain and Fluor chrome staining respectively was 82% and 100%, while specificity of Zihle-Neelson, Fluorochrome respectively were 86% and 100%. Direct examination of sputum samples of patients under treatment and treatment-resistant with mycology staining methods revealed that the fungi were observed in 3 patients (7.1%). Our results showed that the sensitivity of Ziehl-Neelsen stain was lower than Fluorochrome staining, while specificity of Zihle -Neelson was higher than Fluorochrome so, use of both staining methods is needed.

Key words: Fluor Chrome, Ziehl-Neelsen Stain, Multi -Drug Resistance, Pulmonary Tuberculosis Recurrence

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INTRODUCTION

Tuberculosis is a major cause of death and disability in the worldwide. Tuberculosis is a chronic bacterial disease caused by *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*), although in most cases is caused by *M. tuberculosis*. Tuberculosis there is as pulmonary and extra-pulmonary forms. About 85% of TB cases belonged to the pulmonary form and remaining are extra-pulmonary[1]. The World Health Organization (WHO) has been reported that the prevalence of TB in 22 countries around the world more than other countries; Soviet Azerbaijan, Russia, Turkmenistan and the republics of the former Soviet Union, and because of neighborhood of our countries with mentioned countries, we have a difficult situation compared to other countries, for this reason, at now daily incidence rate of TB is reported averagely 7000 deaths (2). It is estimated that every second one people infect with TB. Currently, more than 20 million people are infected with TB worldwide, of which 95% live in developing countries.

It is anticipated that until 2020, tuberculosis is spreading in the world and Still, it has remained as a serious problem if the trend of disease goes like the past; we should be expected annual incidence of 6.5 million cases and more than 4.2 million deaths annually[2, 3].

TB is an endemic disease in Iran and the most of patients are in Sistan and Baluchestan with prevalence more than 25 out of 100000 followed by Golestan province with prevalence 15 to 25 out of 100000[4, 5]. In the patients with MDR-TB which received first-line drugs, but TB is resistant to these drugs and this is a serious problem which increasing in many countries of the world. From 2010 and after that, the number of people with drug-resistant TB (MDR) has risen and the most active age belongs to the age group 15 - 54 years old [6, 7].

Nowadays, MDR tuberculosis is becoming a leading threat to international public health and security, accelerating in recognition of multidrug-resistant *M. tuberculosis* is very important [6, 8]. The re-activation of tuberculosis and emergence of MDR *M. tuberculosis* isolates intensify the necessity for applying the rapid isolation and identification methods for *Mycobacteria*. The major method in the diagnosis of pulmonary

tuberculosis in TB laboratories of developing countries is the smear preparation and direct microscopic observation [9, 10]. Observation of acid-fast bacilli using Zihle - Neelson staining by the microscope is the primary method for diagnosis of TB in the worldwide, but this method doesn't have 100% sensitivity and unable to detect *M. tuberculosis* bacillus in patients undergoing treatment and in TB cases caused by isolates are resistant to treatment. Therefore, more sensitive methods are required for detecting the patients with recurrent TB. So, in addition to available methods, the diagnosis of TB could be confirmed by immunofluorescent staining (Fluor chrome, Calco-flura white), (13). The aim of this study was to identifying and isolation of *M. tuberculosis* isolates from Iranian patients with recurrent TB using different staining methods.

MATERIALS AND METHODS

Population study and inclusion criteria

In this cross-sectional study, from October 2013 to February 2015, during 16 months, 176 sputum samples were collected from patients suspected with TB who were under treatment and TB cases are caused by *M. tuberculosis* isolates resistant to treatment referred to the Health Centre in Sari and Ghaemshahr.

The criteria for inclusion were; adults (male and female), children with respiratory system failure, and patients with underlying conditions such as; defects of the adaptive immune system (HIV), different types of malignancy, COPD and transplant recipients, TB drugs users, cytotoxic and corticosteroid and immunosuppressive drugs consumers, which all patients were examined based on Clinical criteria by lungs specialists and were enrolled in the study.

Clinical Samples Types and Samples collecting method

Samples such as sputum, bronchial, nasopharyngeal washing specimens and every patient suffered from TB who was under treatment and TB cases were caused by *M. tuberculosis* isolates resistant to treatment were entered in the present study. The sputum smears were prepared three times with standard conditions. Samples were collected from 28 positive patients with pulmonary tuberculosis and sampling method was as sequential and patients' specimens collected in Falcon 50 ml containing 2 ml of ethanol 70% and kept in the refrigerator

until transported to the TB laboratory. In order to homogenize the samples with very high viscosity, 4% Sodium hydroxide(NaOH) solution was used and for neutralizing the pH, 1% hydrochloric acid was applied [11].

Laboratory diagnosis of M. tuberculosis using different stainings

sputum samples of TB patients centrifuged at 3000 rpm for 15 minutes, and precipitate prepared, and some of it was used for Ziehl-Neelsen stain, Fluor chrome, calco-Fluor white and GMS stainings and then examined using microscope (optical microscope, immunofluorescence) and a small amount of samples sediment poured on slides of IFA kit and fixed using pathology fixator and kept in the freezer until tested according to the manufacturer's direction and examined by fluorescence microscopy [11].

Data analysis

Data analyzed using *t-test* and descriptive statistics frequency distribution by SPSS Software Version 16. And P-values <0.05 was considered statistically significant.

RESULTS

Of total 176 patients with tuberculosis, 28 cases (15.9%) were smear positive sputum, meanwhile, 17 cases (9.65%) and 11 (6.25%) were male and female, respectively. The mean age of patients was 47±2 years. Twenty-three samples with Ziehl-Neelsen stain staining, 28 samples with Fluor chrome staining were positive for pulmonary tuberculosis (Table1 and Figure 1).

Table 1: Results of stained sputum samples of patients with suspected TB

Test Results	Bacteriology Staining	
	Ziehl-Neelsen stain	Fluor chrome
Positive Result	23	28
Negative Result	5	0
Total	28	28
sensitivity	%82	%100
specificity	%86	%100

Direct examination of sputum samples of patients treated and treatment-resistant with mycology staining methods (GMS and Calco-Fluor white) revealed that the fungi were observed in 3 patients (7.1%) and amongst isolates, *Aspergillus flavus* was the most common agent (Figure 2). The sensitivity of Ziehl-Neelsen stain and Fluor chrome staining respectively were 82% and

100%, while the specificity of Zihle-Neelson and Fluorochrome were 100%, 86%, respectively. As presented in Table 2, there is a significant difference ($p<0.01$) between people suffered from TB in Ziehl-Nelson staining versus to fluorochrome staining.

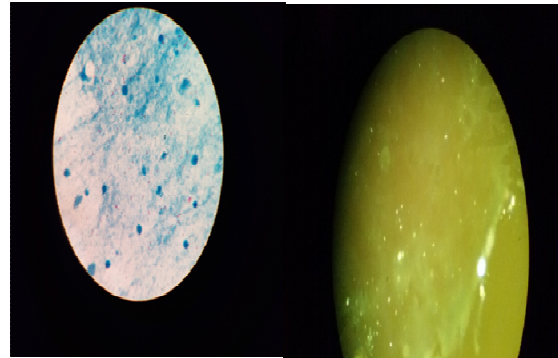


Figure1: Left: Ziehl-Neelsen stain staining. Right: Fluor chrome staining.



Figure 2:Left: GMS staining. Right: Calco-Fluor white staining

Table 2: Table T-test for two methods of coloration Ziehl-Neelsen stain and Fluoro-chrome

Test Results	Mean difference	Standard deviation difference	Levene's test	t	p
Ziehl-Neelsen stain	0.75	0.36	0.012	2.64	0.01
Fluor-chrome	0.96	0.85			

DISCUSSION AND CONCLUSION

Tuberculosis (TB) is a great public health all over the world, and a major step for its control is the prompt and rapid identification of patients, meanwhile, the laboratories have a main role in the diagnosis of pulmonary tuberculosis[12, 13].

It is clear that at present there are diverse laboratory methods for detection of *M. tuberculosis*, but still culture is the gold standard technique for detection of this microorganism and it is a specific and sensitive procedure, but it is a time consuming due to the slow growth of the *M. tuberculosis*, although, at recent years, PCR based methods have been spread because of rapidity, high specificity and sensitivity, nevertheless, these techniques need more intricate laboratory methods and could not be used in diagnostic laboratory, routinely[14]. For these reasons, conventional staining techniques such as Ziehl-Neelsen stain and Fluorochrome have been more developed and attracted more attention.

As mentioned in the results section, the sensitivity of Ziehl-Neelsen stain and Fluorochrome staining methods respectively was 82% and 100%, while the specificity of Ziehl-Neelsen and Fluorochrome were 100% and 86%, respectively. From these data can be concluded that the Fluorochrome has the highest sensitivity and specificity compared to other staining methods in detecting pulmonary tuberculosis in patients with TB resistant to treatment.

In a study conducted by Ziaee and *et al.*, in Birjand(Iran) in 2004, compared Ziehl-Neelsen(ZN) and fluorescence microscopy(FM) methods for detection of bacteria resistant to acid and alcohol, results showed that sensitivity of FM was 2.8 in contrast to ZN[15]. Results of Murray and *et al* were also compatible with our study[16].

In the study carried out by Kivihya Ndugga and van Cleeff in 2003 on 1398 patients suspected of tuberculosis found that in diagnosis of *M. tuberculosis*, FM staining method is more sensitive than Ziehl-Neelsen[17], which their results are consistent with our study, because as mentioned in results section, the sensitivity of Ziehl-Neelsen stain and Fluorochrome staining were 82% and 100%, respectively.

In another study conducted by Githui and *et al.* on 1480 patients revealed that the sensitivity of FM and ZN was 80% and 68%, respectively and also the specificity for two stated staining methods were reported 98% and 96%, respectively[18], which confirms our study, too.

ZN is the simplest and most accessible staining method for laboratory diagnosis of tuberculosis, but due to the need to examine at least 100

microscopic fields with magnification of 100 times, requires precision and a lot of samples of patients for this experiment, while, Auramine-Rhodamine Fluorochrome staining (FM) has the ability to see only 10 fields with a magnification of 10 times, scan the same visual field, with this difference that the time required for this work is a few minutes. FM method features for staining of AFB include: examination capability of smear with lower magnification, the fast scanning capacity of smear within a few minutes, the ability to evaluate the samples with low bacilli that are not recognized by ZN, finally this method is quite time/cost consuming[19-21].

Roya Ghoyal and Anil Kummar in a study conducted in India in 2011 for evaluating the efficacy of fluorescence and Ziehl-Neelsen staining methods in the diagnosis of pulmonary tuberculosis reported that the efficacy (14.69%) of Fluorochrome staining was more than Ziehl-Neelsen with efficacy (7.47%). The highlighted points of our study is that in the above mentioned studies used of clinical samples from patients with suspected pulmonary tuberculosis, but here we used of sputum samples of patients treated and patients with treatment-resistant TB, also in addition to applying bacteriological staining methods, mycology staining methods (GMS and Calco-Fluor white) were used and identified 3 isolates of fungi. In patients treated and treatment-resistant owing to the weakness of the immune system, coinfection of fungi with tuberculosis may be present. For that, according to the medical laboratory guidelines, conventional bacteriological staining methods (ZN and FM) alongside with mycology staining methods should be used to the prevention of missing of possibly fungi agents.

Our results showed that the sensitivity of Ziehl-Neelsen stain and Fluorochrome staining methods respectively was 82% and 100%, while the specificity of Ziehl-Neelsen and Fluorochrome were 100% and 86%, respectively. So, Fluorochrome has the highest sensitivity and specificity compared to other staining methods in detecting pulmonary tuberculosis in patients with TB resistant to treatment. So, use of both staining methods is needed for accurate diagnosis.

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Conflicts of interests

There are no conflicts of interest.

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