

The Comparison between the Modified Auramine Rhodamine Flourochrome Staining and Ziehl-Neelsen Staining Method from Sputum Specimens in the Presence with Radiometric Bactec Tb System

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Abstract

Background: Diagnosis of pulmonary tuberculosis depends on the bacteriological examination of sputum. Sputum smear microscopy is efficient and can confirm the disease. But, direct microscopy of sputum, has low sensitivity. The present study was aimed to evaluate the usefulness of modified – auramine flourochrome staining (MR-AFS) in detection of acid fast bacilli (AFB) from sputum in direct smears (DS).

Methods: A total of 218 patients' specimens 10 positive control and 10 negative control were examined for the presence of AFB by MR-AFS and Ziehl-Neelsen Staining (ZNS) Method in comparison with BACTEC TB system as a gold standard.

Results: 16 specimens were positive for BACTEC TB system and 13 specimens were positive for MR-AFS and 11 specimens were positive for ZNS Method. The detection rate is significantly higher with the MR-AFS than with the ZNS Method ($p<0.01$). When the BACTEC TB system is considered as gold standard the sensitivity and specificity of the MR-AFS were found to be 84% and 100%, while that of ZNS Method were found to be 68.8% and 100%, respectively.

Conclusions: MR-AFS was found to be faster, more practical and more convenient for screening the clinical specimens for AFB than the ZNS Method. In addition MR-AFS is a useful method for screening tuberculosis in all laboratories in developing countries where fluorescent microscope is available.

Key Words: Auramine, stain, tuberculosis, sputum, culture

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ÖZET

Amaç: Akciğer tüberkülozunun teşhisi balgamın bakteriyolojik tetkiki ile mümkündür. Balgam yaymaların mikroskopik incelemesi etkilidir ve hastalığı doğrular. Fakat Balgam yaymaların mikroskopik incelemesinin duyarlılığı düşüktür. Çalışmamızda balgamdan Direkt yaymayla (DS) yapılan preparatların Modifiye– auramine flourokrom boyama (MR-AFS) yöntemiyle aside dirençli basillerin (AFB) boyanmasının kullanışlı olduğunu göstermeyi amaçladık.

Hastalar ve Yöntem: Toplam 218 hastadan 10 pozitif kontrol ve 10 negatif kontrolden alınan AFB'in MR-AFS ve Ziehl-Neelsen Boyama ZNS yöntemleri ile BACTEC TB sistem altın standard olarak kabul edilerek incelendi ve kıyaslandılar.

Bulgular: BACTEC TB sistemi ile 16 örnek, 13 örnek MR-AFS yöntemi ile ve 11 örnek ZNS AFS yöntemi ile pozitif olarak tespit edilmiştir. Pozitif olarak tespit edilen örneklerin oranları ZNS yönteminde MR-AFS yöntemine göre belirgin olarak yüksek olarak bulunmuştur ($p<0.01$). BACTEC TB sistem altın standard olarak kabul edildiğinde MR-AFS'nin sensitivite ve spesivitesi sırasıyla %84 ve %100, ZNS'nin sensitivite ve spesivitesi sırasıyla %68.8 ve %100 olarak tespit edilmiştir.

Sonuç: Klinik örneklerden AFB araştırılmasında MR-AFS yönteminin ZNS yöntemine göre daha hızlı pratik ve güvenli olduğu tespit edilmiştir. Ayrıca gelişmekte olan floresan mikroskopunun olduğu ülkelerde MR-AFS'nin kullanışlı bir yöntemdir

Anahtar kelimeler: Auramin, boyama, tüberküloz, balgam, kültür

Introduction

Tuberculosis, caused by the intracellular pathogen *Mycobacterium tuberculosis*, is the world's leading cause of death from a single infectious agent in humans^{1,2}. The principal etiologic agent, *M. tuberculosis*, currently infects two billion people worldwide and causes eight million new cases of active tuberculosis and 2.9 million deaths annually³. The emergence of multidrug-resistant strains of tuberculosis is an ominous new threat to public health^{1,4}.

Diagnosis of mycobacterial infections is still a long process that traditionally depends on the isolation of the pathogen *M. tuberculosis* which can take up to six to eight weeks because of the slow-growing nature of mycobacteria. There are lots of new and conventional methods used for the diagnosis of mycobacterial infections. Some of them are used as gold standart. The BACTEC system (Becton-Dickinson Diagnostic Instrument Systems, Sparks, MD.), recent development for the

rapid detection of mycobacteria based on radiometric monitoring, has added a new dimension to diagnostic microbiology. This method has reduced the turnaround time for isolation of AFB to approximately 10 days, compared with 17 days for conventional media^{5,6}. The BACTEC tuberculosis system is based on the principle that the organisms multiply in the broth and metabolize ¹⁴C-containing palmitic acid, releasing radioactively labeled ¹⁴CO₂ that collects above the broth in the bottle into the atmosphere. The BACTEC instrument withdraws this ¹⁴CO₂-containing atmosphere and measures the amount of radioactivity present. Those bottles that yield a radioactive index, called "growth index" greater than 10 are considered to be positive. The results of obtained from these methods compared with that of conventional method indicate that the BACTEC agrees very well with conventional results^{2,4}.

The staining methods vary between ZNS Method and MR-AFS Method. The fluorochrome staining method is recommended for those laboratories that poses a fluorescent (ultraviolet) microscope. This stain is more sensitive than the conventional carbolfuchsin stains because the fluorescent bacilli stand out brightly against the background. Also the smear can be initially examined at lower magnifications (250x to 400x). Therefore more fields can be visualized in a short period of time. More over, many laboratory workers use the – auramine staining for acid-fast bacilli (AFB) in primary specimen smears rather than fuchsin-based acid-fast methods because the former method of staining is more readily interpreted and yields greater sensitivity than the latter methods^{2,7-10}.

The aim of this study was the detection of AFB in direct smears from sputum stained with the MR-AFS method. Furthermore, this study compared the results from this modified -auramine staining method with the results from the conventional Ziehl-Neelsen Method in the presence of radiometric BACTEC tuberculosis system as gold standard.

Materials and Methods

Specimens: A total of 218 sputum specimens from the patients admitted to University Hospital at Gaziantep University in Turkey was cultivated with BACTEC TB system, at Department of Microbiology and Clinical Microbiology. Then sputum specimens were examined at the Çukurova University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Adana / Turkey. During this process, specimens were first decontaminated and digested with NALC and NaOH for 15 minutes. Finally centrifugation was then performed three times at 3000 xg for 15 minutes and the sediment was resuspended in

sterile phosphate buffer to a total volume of approximately 2 ml.².

Slide Preparation:

1) Modified MR-AFS fluorochrome staining (MR-AFS) method:

For direct smears, one slide was prepared from each specimen. As positive control, known positive specimens graded as 1+, 2+, 3+, 4+ were processed and smears were prepared from each. The control smears were stained separately to avoid cross contamination in a different dish and on a staining tray for the modified -auramine method. In addition, concentrated smears were prepared from each specimen and stained with modified MR-AFS^{10,11}.

2) The Ziehl-Neelsen staining (ZNS) method:

The DS for this method were prepared in the same way as the DS for the rodamine-MR-AFS fluorochrome staining method. The DS were prepared on individual slides for each specimen. Positive and negative controls were used in the same manner mentioned above.

Staining Methods:

1) MR-AFS method:

This method is a combination of the MR-AFS fluorescence technique of Kuper and May and the van Gieson stain was performed. It was used according to the procedure^{10,12}. Each time this MR-AFS method was performed (sigma diagnostics, St Luis, USA). Sputum smear with a large number of acid-fast bacilli was also used as a quality control of the staining method.

2) The Ziehl-Neelsen staining method:

It was used according to the procedure².

Interpretation of the slides

Direct and concentrated specimen smears were interpreted without knowledge of the other staining method results. A light microscope was used for the interpretation of slides stained with the ZNS Method at 100X oil immersion objective (1000X magnification). Fluorochrome stained smears were scanned at 100X to 200X magnification with fluorescence microscope, with confirmation of AFB morphology at 400X and 1000X magnifications. The presence of the characteristic orange-colored fluorescing rods was screened first under low power then, if present, under 100X objective was screened for confirmation.

Positive smears were graded according to the Kent and Kubica's criteria⁹:

Dubfull, repeat, 1 to 2 organisms per 20 30x fields,
1+, rare, 1 to 9 organisms per 10 20x fields,
2+, few, 1 to 9 organisms per field
3+, moderate, 10 to 90 organisms per field
4+, numerous, >90 organisms per field

The slides which were graded as doubtful for the MR-AFS method were then stained with the ZNS Method for confirmation². All the slides which were positive and negative with the MR-AFS method, were stained with ZNS Method for confirmation.

Determination of sensitivity and selectivity of modified MR-AFS staining:

Modified MR-AFS staining was compared with the conventional Ziehl-Neelsen Staining in detecting AFB in sputum. The detection rate was significantly higher with the MR-AFS than with the ZNS ($p < 0.01$). The sensitivity and selectivity of the modified MR-AFS staining were found to be 100 % and 93.4 % respectively.

BACTEC method:

After the specimen was digested and decontaminated using NALC and NaOH, the sediment was resuspended in sterile phosphate buffer to a total volume of approximately 2 ml. A small quantity (0.4 to 0.5 ml) of this suspension was injected into a BACTEC bottle containing 7H12 medium with added antimicrobial agents. The rest of the sediment was used for conventional smear and culture. Cultures were incubated at their places, and BACTEC bottles were incubated in air at 35°C. The bottles were tested for ¹⁴CO₂ production three times weekly for 6 weeks. Bottles that register a growth index greater than 50 were split into two samples, each of which was injected into a fresh bottle or medium. A 5-µg quantity of *p*-nitro- α -acetylamino- β -hydroxypropiophenone (NAP) was added to one of the bottles, and they were both reincubated. These two bottles were then tested on the BACTEC daily for 4 days. An increase in the growth index in the unaltered control bottle without a corresponding increase of the growth index in the bottle containing NAP indicated the presence of *M. tuberculosis* or *M. bovis*, both of which were susceptible to NAP. 10 positive and 10 negative control samples was observed and cultured with the other sputum samples and analyzed after research.

Statistics

For the statistical analysis McNemar's χ^2 test was carried out using the Epi info 6 computer program¹³.

Results

Sputum specimens from 218 patients, 10 positive samples and 10 negative samples (total 238 samples) were examined for the presence of AFB using the MR-AFS, DS. The ZNS method was compared with the MR-AFS method for DS. The results with samples were as follows: 16 specimens (7.3 %) were positive for BACTEC TB culture

system, 13 specimens (6 %) were positive for MR-AFS method of DS smears and only 11 (5 %) were positive with the ZNS of DS. BACTEC culture method captured all positive and negative controls. ZNS was excellent for finding negative controls but failed to find 3 of 10 positive samples. MR-AFS was also excellent for finding negative controls but failed to find 1 of 10 positive samples. All positive results of samples, positive and negative controls results of samples are given in Table 1.

METHOD	Positivity	10 positive control result	10 negative control result
Ziehl-Neelsen staining	11	7	10
Modified – auramine staining	13	9	10
BACTEC TB culture	16	10	10

Table I: Results of samples with Ziehl-Neelsen staining, Modified – auramine staining and BACTEC TB culture

Sensitivity and Specivity of two staining and BACTEC TB culture method (for 10 positive and 10 negative control's and sample's are given in table 2.

METHOD	Control's	Sample's
Sensitivity of Ziehl-Neelsen staining	70%	68.8%
Specivity of Ziehl-Neelsen staining	100%	100%
Sensitivity of Modified – auramine staining.	90%	81.3%
Specivity Modified – auramine staining	100%	100%

Table II: Sensitivity and Specivity rates of Ziehl-Neelsen staining and Modified – auramine staining

If we consider BACTEC TB culture method as gold standard, the real positive, real negative, false positive and false negative results for staining methods are given in table 3.

The detection rate with MR-AFS was significantly higher than that of with the ZNS. (McNemar's χ^2 , $p < 0.01$). When we consider BACTEC TB culture method as gold standard, the sensitivity and specificity of the ZNS were found to be 69.2% and 100% and the sensitivity and specificity of the MR-AFS were found to be 84% and 100%, respectively.

The largest zone of inhibition was found with Klorhex oral gel (control group). But one-way ANOVA indicated no significant differences between Klorhex oral gel, Calcicure and Ca(OH)₂ powder mixed with Klorhex irrigation solution. There was no change in the antibacterial activity of all of the sealers except that Sealapex from the 24-h

period through the 48-h period and 24-h period through the 72-h period. From the 48-h period through the 72-h period, there was no change in the antibacterial activity of all of the sealers.

<i>Ziehl-Neelsen staining</i>	<i>Sample's</i>
Real positive (+C; +ZNS)	11
Real negative (-C; -ZNS)	202
False positive (-C; +ZNS)	0
False negative (+C; -ZNS)	5
Sensitivity (RP) / (RP+FN)	68.8%
Specivity (RN) / (RN+FP)	100%
Modified – auramine staining	
Real positive (+C; +ZNS)	13
Real negative (-C; -ZNS)	202
False positive (-C; +ZNS)	0
False negative (+C; -ZNS)	3
Sensitivity (RP) / (RP+FN)	81.3%
Specivity (RN) / (RN+FP)	100%

Table III: When BACTEC TB culture method considered as gold standard, the real positive, real negative, false positive and false negative results for staining methods

C: + BACTEC TB Culture RP: Real positive RN: Real negative FP: False positive FN: False negative

Discussion

Tuberculosis is one of the world's deadliest infectious diseases^{1,2}. Despite many recent advances in mycobacteriology, early laboratory diagnosis of tuberculosis still relies heavily upon the examination of stained smears. Not only it is still the easiest, cheapest, and most rapid procedure, but also provides the physician with important preliminary information. Depending largely on the extent of the lesion, the overall sensitivity for microscopy is only between 22 and 65%¹⁴. The Ziehl-Neelsen method for staining direct smears of clinical specimens lacks sufficient sensitivity and specificity. This is due in part to underlying problems including unqualified staff and a lack of time needed for screening, and a lack of patience to observe under the microscope¹⁵. Mycobacteria might escape the detection by microscopic examination with the Ziehl-Neelsen staining but can be detected very easily by MR-AFS staining². Some authors reported that they routinely use a slightly modified version of Hagemann's auramine stain for the demonstration of AFB in cases of suspected tuberculosis in unfixed clinical materials as well as formalin fixed and paraffin-embedded material^{7,16}. The theoretical basis of this method is

practically the same as that of the ZNS^{7,8,16}. It undoubtedly works well on any kind of clinical specimen. As with all chemicals used for stains, there are occupational hazards reported with phenol and auramine^{7,8}. In this study, the ZNS was compared with the MR-AFS from DS in the presence of BACTEC method as gold standard. 16 specimens (7.3%) were positive by BACTEC method. 11 specimens (5%) were positive by the ZNS of DS, and 13 specimens (6%) were positive by the MR-AFS of DS (Table 1). When the BACTEC method will considered as gold standard ZNS and the MR-AFS may be compared better.

In many studies the MR-AFS method and MR-AFS staining procedure has been shown very good performance when comparing with the other staining procedures^{2,8,15}. McCarter and Robinson studied a total of 782 concentrated primary specimen smears stained with MR-AFS staining at room temperature and at 37°C and found that 30 of the smears were positive for AFB at room temperature and 35 were positive at 37°C. They concluded that staining at 37°C worked better. In this study MR-AFS at 37°C gave greater sensitivity in detecting AFB in clinical specimens. The current study also supported this finding that MR-AFS at 37°C gave greater sensitivity in detecting AFB in clinical specimens¹⁷.

Van der Zanden et al. reported that paraffin wax embedded tissue samples and Ziehl-Neelsen and auramine stained microscopic preparations from culture positive tuberculosis patients were subjected to DNA extraction and amplification by PCR. As a result of the study, detection of tuberculosis from paraffin wax embedded tissue samples with auramine stained microscopic preparations was available¹⁸.

In contrast, Collins et al. studied with standard smears of heat-killed Mycobacterium leprae and M. tuberculosis H37Rv which were counted microscopically following staining by the Ziehl-Neelsen, auramine, and silver-methenamine methods. They found that the numbers of stained bacillary bodies were consistently higher in the silver-methenamine stained smears compared to the Ziehl-Neelsen and auramine stained smears. Collins et al. pointed that the auramine stained smears were examined under ultraviolet illumination and permitted the enumeration of the brightly fluorescent bacilli against a black background. In that study the auramine counts were not as high as those obtained using the silver-methenamine stained preparations but were consistently higher than those obtained with the Ziehl-Neelsen preparations¹⁹.

Cserni et al., pointed out that he routinely uses a slightly modified version of Hagemann's auramine stain for the demonstration of acid-fast bacilli in

cases of suspected tuberculosis on formalin-fixed and paraffin-embedded material⁷. He found that it worked better than the previously used ZNS. In contrast to these recent studies, some authors believe that classic auramine fluorescence is usually restricted to unfixed material such as cytologic specimens^{7,8,16}).

Nevertheless, when these methods are used, mycobacterium might escape the detection by microscopic examination, but can be amplified by PCR². However, the false positivities of PCR should be kept in mind².

In daily routine work, the primary diagnosis is made only with ZNS. In negative cases or cases suspicious for nontuberculous mycobacterial infection, the MR-AFS should be used as a second stepwise diagnostic procedure.

Wöckel reported the advantages of the combined staining technique (the auramine fluorescence technique of Kuper and May and the van Gieson stain) and used this technique successfully daily for many years^{1,10,20}. He also compared the ZNS with the auramine stain and found the latter better, more rapid, and, most importantly, more sensitive for AFB. In addition there are many studies comparing ZNS with the auramine stain. In these studies the auramine stain found to be superior to ZNS²¹⁻²⁷. The present study showed that sensitivity of MR-AFS (84%) is higher than ZNS (68.8%) method in presence with BACTEC TB culture method.

In the study carried out in Australia, 162 cultures were examined using BACTEC TB culture method and Löwenstein - Jensen culture method. When the results compared it was found that BACTEC TB culture giving 147 (91%) positive results, was more sensitive than Löwenstein - Jensen culture method giving 118 (73%) positive results²⁸.

It was also found the MR-AFS to be superior when compared to the ZNS²⁹. It was determined that the sensitivity and selectivity of the MR-AFS were to be 100 % and 93.4 % respectively. Although both MR-AFS and the ZNS methods are classical staining methods, the MR-AFS can be and should be used interchangeable²⁴. In addition to the results mentioned above, the current study revealed the following advantages of MR-AFS method over ZNS were also determined the advantages of this method.

1. Less time is required for AFB detection. Thereby saving the time needed to make a fresh smear.
2. This method provides a greater sensitivity for AFB.
3. Moreover, it is possible to stain the MR-AFS stained preparation with the ZNS after-wards.

In conclusion, the staining methods of DS showed that the MR-AFS was sensitive (100 %) and selective (84%) in detecting mycobacteria. The results of this study showed that the MR-AFS at 37°C enhanced the detection of AFB as compared with the ZNS method. It may also become a useful tool in the early and rapid detection of mycobacterial infections. When performed on DS smears, the modified MR-AFS method was found to be faster, more practical, and more convenient for screening the clinical specimens for AFB than the Ziehl-Neelsen method. The results of present study which are in congruence with the findings in the auramine staining with MR-AFS method is more sensitive than ZNS, but finding fluorescent microscope isn't available in developing countries all the time²². Thus, it is recommended that when fluorescent microscope is available, auramine method should be used for detection of mycobacteria especially from sputum samples.

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