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A comparison of two different fluorochrome stains for the detection of acid-fast bacilli in sputum specimens*

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Aim: The early diagnosis of active tuberculosis still depends on the presence of acid-fast bacilli (AFB) in stained sputum smears. In this study, our aim was to investigate the efficiency and cost-effectiveness of two different fluorochrome stains.

Materials and methods: A total of 1013 sputum specimens were collected from 642 patients. Three smears and cultures were prepared from each specimen. Double-blind and prospective laboratory procedures were performed. Slides were stained with a commercial auramine/acridine orange kit (Stain 1), an in-house preparation of auramine- rhodamine/KMnO4 (Stain 2) and a Ziehl-Neelsen stain (EZN).

Results: Of the 1013 specimens, 101 were culture positive. Among these, AFB was detected in 60 specimens by EZN, in 53 by Stain 1, in 81 by Stain 2. By cultures, the sensitivities and specificities of Stain 2 were 80.1% and 83.8%, respectively, and for Stain 1, 52.4% and 94.6% respectively. There is no significant difference between the costs of these methods.

Conclusion: Stain 1 was easy to apply and inexpensive but the sensitivity of Stain 1 was lower than that of Stain 2. However, Stain 2 required longer preparation time, more work, and had a higher risk of exposure to carcinogens. In order to increase the sensitivity of Stain 1, it is suggested that the contents of the prepared Stain 1 kit could be rearranged. In tuberculosis diagnosis, this revised kit may provide practicality in use.

Key words: Mycobacteria, tuberculosis, auramine/acridine orange kit, auramine- rhodamine/KMnO4, fluorochrome stain

Balgam örneklerinde aside dirençli basil saptanması için iki farklı florokrom boyanın karşılaştırılması

Amaç: Aktif tüberkülozun erken tanısı hala balgam yaymalarında aside dirençli basil varlığına bağlıdır. Bu çalışmada amacımız iki farklı florokrom boyama yönteminin maliyetini ve etkinliğini araştırmaktır.

Yöntem ve gereç: 642 hastadan 1013 balgam örneği toplandı. Her örnekten üçer yayma preparat ve kültür yapıldı. Laboratuvar yöntemleri çift kör ve prospektif olarak uygulandı. Yayma preparatlar, ticari Auramine/Acridine Orange kit (Boya1), manuel olarak hazırlanan Auramine- Rhodamine/KMnO4 (Boya 2) and Ziehl-Neelsen stain (EZN) boyaları ile boyandı.

Bulgular: 1013 balgam örneğinin 101'i kültür pozitifti. Kültür pozitiflerin 60'ında EZN, 53'ünde Boya1 ve 81'inde Boya2 ile aside dirençli basil saptandı. Kültüre göre boya1 ve boya2 nin duyarlılık ve özgüllükleri sırasıyla % 52,4, % 94,6 ve % 80,1, % 83,8 dir. Boyama yöntemlerinin maliyetleri arasında istatistiksel anlamlı fark saptanmadı.

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Sonuç: Boya1'in duyarlılığı Boya2'den daha düşük fakat daha kolay uygulanabilir ve ucuzdur. Bununla birlikte Boya2'nin hazırlık dönemi daha uzun zaman gerektirir, iş gücü fazladır ve kansorejen madde ile maruziyet riski daha fazladır. Boya1'in duyarlılığını arttırmak için içeriğinin yeniden gözden geçirilmesi önerilir. Tüberküloz tanısında yeniden düzenlenen kit içeriği pratik kullanım sağlayacaktır.

Anahtar sözcükler: Mikobakteri, tüberküloz, auramine/acridine orange kit, auramine- rhodamine/KMnO4, florokrom boyama

Introduction

In many countries, tuberculosis (TB) is still one of the most problematic diseases. According to the World Health Organization's (WHO) 2009 report, the incidence rate of TB in Turkey was 30 (24-36) per 100,000 population in 2008 (1). Accurate detection is the rate-limiting step in TB control and TB laboratories are essential for definitive diagnosis (2,3). In spite of the numerous diagnostic methods, the early diagnosis of active tuberculosis still depends on the presence of acid-fast bacilli (AFB) in stained smears. The specificity of AFB microscopy is high but its sensitivity varies. The sensitivity of AFB microscopy is influenced by diverse factors such as the prevalence of tuberculosis, the quality and number of specimens, the staining technique, the experience of the microbiologist, etc. In addition, there are differences between the types of staining methods in terms of specificity, sensitivity, and cost (4). As for the staining techniques, the sensitivity of fluorochrome staining (FS) is higher than that of carbol-fuchsin methods such as Erlich-Ziehl-Neelsen (EZN) or Kinyoun, which are the most commonly used techniques. Fluorochrome microscopes are more expensive than conventional microscopes. However, recently developed lower-cost light-emitting diode (LED) fluorochrome microscopes are expected to decrease the cost burden resulting from the use of traditional fluorochrome microscopes. These new microscopes are scheduled to be comprehensively evaluated for routine use in high-burden countries (5,6). Considering cultures to be the gold standard in diagnostic method, we aimed to compare the sensitivity and cost efficiency of 2 fluorochrome stains. EZN staining was used for final confirmation.

Study population and methods

Setting and sampling

The sputum specimens included in the study were obtained from patients admitted to Kahramanlar Regional Tuberculosis Laboratory (KRTL) located in İzmir, the third largest city in Turkey. The laboratory accepts materials from 28 tuberculosis dispensaries in the Aegean region and between 15,000 and 18,000 sputum samples are processed by the laboratory each year. In this laboratory, fluorochrome staining and drug susceptibility tests are not performed routinely. In the present study, the sputum samples obtained from KRTL were prepared using the concentration technique. They were stained for AFB examination using EZN and cultured in Löwenstein-Jensen medium (LJ). The study was conducted over a three-month period in 2005. A total of 3420 specimens in standard specimen containers were accepted for routine AFB detection during the study period. 1013 sputum specimens were chosen from 642 patients by random sampling. From each container, at least 2 mL of the sputum specimen was used for examination.

Laboratory procedures

The laboratory procedures performed were double-blind and prospective. Three smears were prepared from each sputum specimen. Slides were stained with a commercial auramine kit (Stain 1), [auramine/acridine orange (ST 022, Salubris)]; a fluorochrome stain prepared in-house (Stain 2), [auramine-rhodamine / $KMnO_4$], and Ehrlich-Ziehl-Neelsen (EZN) stain. The contents of the acid-fast stains and the details of the staining procedures used in the present study are given in Tables 1 and 2. Stain 1 is a fluorochrome stain kit which is commercially available in Turkey at the present time. However, since 2002, the Tuberculosis Working Group (TWG) of the Turkish Society of Clinical Microbiology and Infectious Diseases has been running a postgraduate

Table 1. The contents of the acid fast staining methods used in the study.

Carbol-Fuchsin Staining	Fluorochrome Staining	
Ehrlich-Ziehl-Neelsen (EZN)	Commercial auramine kit (Stain 1)	In-house prepared fluorochrome stain (Stain 2)
	Auramine/acridine orange (ST 022, Salubris)	Auramine-rhodamine / $KMnO_4$
Solution I Basic fuchsin*------- 0.3 g Ethanol 95%------- 10 mL Solution II Distilled water------10 mL Phenol**---------- 5 g Mix: Solution I ----10 mL Solution II ----90 mL	Auramine solution	**Mix 1:** Auramin O (Sigma 9655)---- 1.5 g Rhodamin B(Merck) -------- 0.75 g Glycerol ----------75 mL Phenol ---------- --------10 mL Distilled water----------50 mL
Acid-Alcohol (3%)	Acid-alcohol (0.5%)	Acid-alcohol (0.5%)
Methylene blue chloride*** 0.3 g Distilled water -------100 mL	Acridine orange solution	**Mix 2:** Potassium permanganate (Merck) 0.5 g Distilled water ---------- 100 mL

* Basic Fuchsin (Merck, ZC 262237-548)
** Phenol (Merck, KA 24980945-812)
*** Methylene blue chloride (Merck, ZA 2620500-837)

Table 2. Acid fast staining procedures.

Carbol-Fuchsin Staining	Fluorochrome Staining	
Ehrlich-Ziehl-Neelsen (EZN)	Commercial auramine kit (Stain 1)	In-house prepared fluorochrome stain (Stain 2)
	Auramine/acridine orange (ST 022, Salubris)	Auramine-rhodamine / $KMnO_4$
* Flood the slide with carbol-fuchsin * Heat the slide slowly for 3-5 min until it is steaming – Do not boil * Rinse the slide with sterile water * De-colorize for 2 min * Rinse the slide with sterile water * Flood the slide with methylene blue for 30 s * Rinse the slide with sterile water * Air dry * Examine the smear with microscopy	* Flood the slide with auramine * Stain for 15 min * Rinse the slide with sterile water * Flood with acid-alcohol for 30-60 s * Rinse the slide with sterile water * Flood the slide with acridine orange for 2 min * Rinse the slide with sterile water * Air dry * Examine the smear with microscopy	* Flood the slide with Mix 1 * Stain for 15 min * Rinse the slide with sterile water * Flood with 0.5% acid-alcohol * De-colorize for 2 min * Rinse the slide with sterile water * Flood the slide with potassium permanganate for 2 min * Rinse the slide with sterile water * Air dry * Examine the smear with microscopy
Positive: Red-stained rods on blue background Negative: No red-stained rods observed	Positive: Orange to red fluorescence on black background Negative : No fluorescence	Positive: Yellow to orange fluorescence on black background Negative : No fluorescence

course on TB diagnosis and Stain 2 is used as the primary recommended method for the diagnosis of tuberculosis in that course.

Stain 1 is a commercial auramine kit and the stain solution only needs to be shaken carefully for a few minutes. In preparing Stain 2, however, each chemical was weighed separately and the solution mix was prepared in a biosafety cabinet. The solution was subsequently filtered and kept waiting for 18-20 h. The preparation time for Stain 2 was approximately 24 h. During the preparation phase, all protective measures were taken to minimize the carcinogenic effect. Facial masks, gloves, eyeglasses, and gowns were worn as personal protective equipment.

In the present study, EZN stained smears were examined using the ×100 oil immersion objective (×1000) and fluorochrome stained smears were examined using the ×40 objective (×400) under a fluorescence microscope. With the auramine/acridine orange stain, mycobacteria appear bright yellow ; using the auramine-rhodamine / $KMnO_4$ stain, they appear orange-red against a dark background (Figures 1 and 2). All slides identified as positive by fluorochrome stains (Stain 1 and 2) were re-stained with EZN. At the beginning of each day of the study, the positive and negative samples were stained. All sputum specimens were cultured on LJ medium. After the evaluation of both culture results and the clinical condition of the patients, samples deemed to be false positive or false negative were excluded. Specificity, sensitivity, and negative and positive predictive values were calculated for each method according to the culture results. The costs of the 2 fluorochrome staining methods were also compared.

Cost analysis

The price of the ready-made kit used for Stain 1 was recorded as the final cost. The staining preparations for Stain 2 were started at least 24 to 36 h before the staining process. As the second stain required in-situ preparation, the prices of the chemical substances needed to prepare Stain 2 were added. For both stains, the cost of the amount of stain used for the staining of all samples was calculated by multiplying the price per unit. The cost of equipment such as glass slides, which were used for both staining methods, was excluded from the calculation of cost. The costs required to prepare 100 slides with each method were determined and compared.

Statistical methods

After the data on each specimen were loaded into the SPSS 12.0 statistic program, the results were interpreted. Chi square test, odds ratio, and kappa statistics were used for statistical analysis.

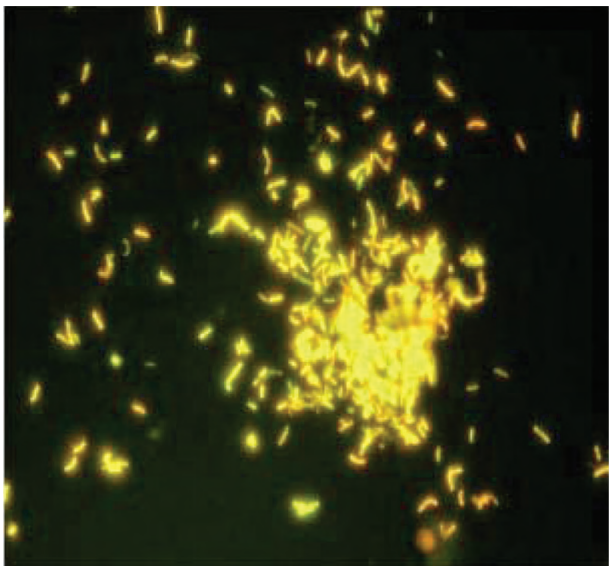

Figure 1. Fluorochrome stain prepared in-house, auramine-rhodamine/$KMnO_4$.

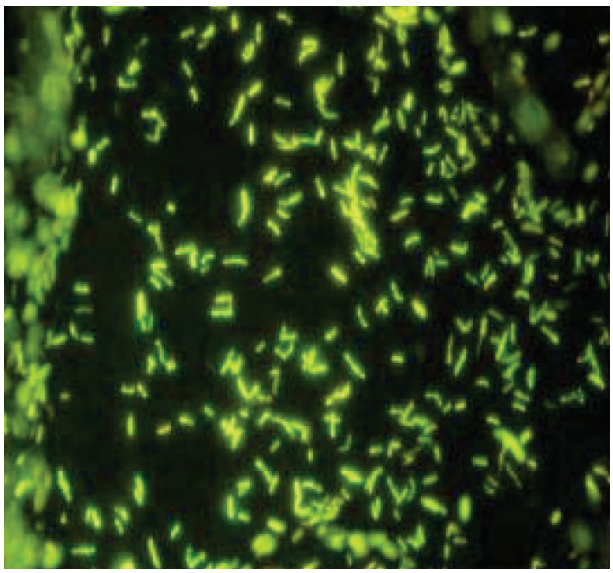

Figure 2. Commercial auramine kit, auramine/acridine orange (ST 022, Salubris).

Results

Of the 1013 sputum samples, 460 (45.4%) belonged to individuals who applied to our department to receive health certificates for job applications, 333 (32.9%) belonged to patients receiving TB treatment and follow-up care, 148 (14.6%) belonged to patients whose treatments were finalized and who were scheduled to receive follow-up monitoring for 2 years without medication, and 72 belonged to tuberculosis (7.1%) contacts. Among 1013 specimens, 101 (9.97%) were culture positive and of these specimens, AFB was detected in 60 using EZN, in 53 using Stain 1, and in 81 using Stain 2. The relationship between the results of the culture samples and staining methods is presented in Table 3 and Figure 3.

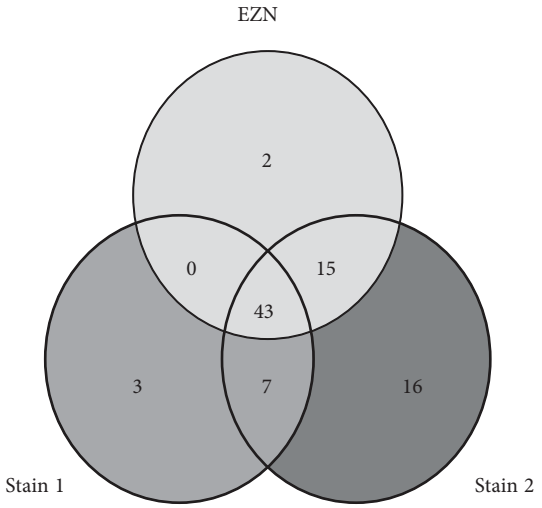

Figure 3. The results of the staining methods in culture positive sputum samples.

The preparation of Stain 1 takes only a few minutes whereas the preparation of Stain 2 should be started at least 24 h before use. After these preparations, the staining process for both methods takes approximately 20-25 min. The culture reliabilities of both staining methods for the whole group were evaluated individually using kappa analysis. It was determined that the culture reliability was moderate for Stain 1 and Stain 2 (kappa > 0.42). The reliability between Stain 1 and Stain 2 was low (kappa = 0.27). As for the culture positive group, the reliability of both staining methods was moderate (kappa = 0.55). The EZN stain reliabilities of Stain 1, Stain 2, and cultures for the whole group were determined as moderate (kappa = 0.51), low (kappa = 0.38), and high (kappa = 0.64), respectively.

Specificities, sensitivities, and negative and positive predictive values of the methods according to the culture results are shown in Table 4. The results of the cost analysis were €15.30/100 slides for the commercial auramine kit, and €17.80/100 slides for the fluorochrome method prepared in-house.

Discussion

After the evaluation of the medical history and a clinical assessment of the patient, the first step in the diagnosis of TB is determining that it may be a possibility. This preliminary diagnosis is supported by radiological findings and PPD results. However, the definitive diagnosis can only be made if the TB bacillus can be found in clinical samples. Especially in developing countries, the early diagnosis and treatment of active pulmonary tuberculosis still depends on the presence of AFB on stained smears (7). In order to show the bacillus, the sample collected from the patient who is at risk for tuberculosis or

Table 3. The results of the acid fast staining methods according to the culture.

	EZN		Stain 1		Stain 2	
	n	(%)	n	(%)	n	(%)
Smear (+), Culture (+)	60	(5.92)	53	(5.24)	81	(7.99)
Smear (+), Culture (-)	15	(1.48)	61	(6.02)	147	(14.52)
Smear (-), Culture (+)	41	(4.05)	48	(4.74)	20	(1.97)
Smear (-), Culture (-)	897	(88.55)	851	(84.00)	765	(75.52)
Total	1013	(100)	1013	(100)	1013	(100)

Table 4. Specificity and sensitivity rates and negative and positive predictive values of the staining methods.

Staining methods		Culture results (n)			Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Positive	Negative	Total				
Stain 1	Positive	53	71	124	52.4	**94.6**	**42.7**	94.6
	Negative	48	841	889				
	Total	101	912	1013				
Stain 2	Positive	81	147	228	**80.1**	83.8	35.5	**97.4**
	Negative	20	765	785				
	Total	101	912	1013				
EZN	Positive	60	15	75	59.4	**98.3**	**80.0**	95.6
	Negative	41	897	938				
	Total	101	912	1013				

suspected to have tuberculosis should first be properly delivered to a laboratory. Additionally, the use of high sensitivity acid-fast staining methods increases the chance of a correct diagnosis. According to previous studies, the sensitivity of the EZN stain ranges between 32% and 94% when used in routine practice and fluorescence microscopy is approximately 8-10% more sensitive than EZN microscopy (4,8). In one study, it was stressed that when the number of samples is limited fluorochrome staining should be used (9). The number of specimens in the present study was similarly limited to 1 or 2 per patient. The sensitivities of EZN and Stain 1 were found to be similar and the sensitivity of Stain 2 was found to be higher than the other 2 stains when cultures were considered as the gold standard. False positive results are expected outcomes when methods with high sensitivity are employed and it should be noted that false positive results in flurochrome staining methods have also been reported (10,11). Since all slides where AFB were detected by fluorochrome stains were also confirmed by EZN staining, false positive results can be corrected. The high reliability between EZN and cultures determined by the present study stresses that the sputum samples detected as positive by fluorochrome staining should be absolutely confirmed by EZN. However, as the use of fluorochrome stains increase false negative results, the possibility of detecting positive patients decreases. Moreover, there is no opportunity to re-evaluate the slides which are found to be negative by fluorochrome staining.

In the present study, instead of staining all 1013 sputum samples with EZN, only 228 were stained for confirmation when the highly sensitive Stain 2 was used first. As for Stain 1, the confirmation staining of 124 samples with EZN was sufficient. In laboratories which accept and test a great number of sputum samples(like the laboratory where our study was conducted, which tests 65-70 samples per day), it is extremely difficult to test all of the samples with EZN and provide accurate results to the clinics in 24 h. Researchers can save time by using EZN only to confirm samples previously found to be positive by a high sensitivity method like FS which yields faster initial results than EZN.

The sensitivity of Stain 2 was higher than Stain 1 and false negative results obtained with this stain were lower than with Stain 1. Thus, it can be concluded that applying a test with a staining method with high sensitivity results in a decrease in the number of slides to be examined, shortens the test duration for each slide, and increases the possibility of detecting culture-positive samples. The detection of positivity in 81 of the 101 culture-positive sputum samples supports this recommendation. As for Stain 1, which demonstrated lower sensitivity and a higher rate of false negatives than Stain 2, its sensitivity can be increased and its false negative result rate can be decreased by revising the kit's contents and replacing the contrasting acridine orange stain with $KMNO_4$. This revised kit, which would have a higher sensitivity than EZN staining, might be able to provide practicality in routine use.

It has been reported that there are minor differences in the sensitivity and specificity of fluorochrome staining methods (4,12). Considering the reliability of results observed between cultures and the staining methods of the present study, it can be concluded that the low to moderate reliability of both staining methods when compared with cultures or with each other and the high reliability of EZN with cultures emphasize that the slides showing a positive result should be checked again with EZN staining after de-colorization, regardless of which type of fluorochrome staining is used first.

Additionally, although no significant difference was observed in the costs of the stains, by taking into consideration the time spent on stain preparation (at least 24 h) and the accompanying risk of cancer, it can be concluded that the use of a commercial kit with high sensitivity and a low rate of false positive would help to ensure that the results are available to the clinics within 24 h. According to the International Agency for Research on Cancer (IARC)'s monographs on the evaluation of carcinogenic risks to humans, auramine was classified in the Group 2B which includes agents which are possibly carcinogenic to humans. Although the relationship between exposure to auramine and risk of cancer has not yet been proven, auramine is thought to be associated with an increased incidence of bladder and prostate cancer (13).

In conclusion, the fluorochrome staining methods used for TB screening can decrease the workload in laboratories which have to test a great number of slides every day. Although there are minor differences between the staining methods, the detection of TB in a short period of time by using a staining method with both high sensitivity and high specificity can increase the success of tuberculosis control programs.

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