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Lab dx - microscopy fluorescence

FLUORESCENCE MICROSCOPY OF TUBERCLE BACILLI STAINED WITH AURAMINE AND RHODAMINE

TRUANT, J.P.,* BRETT, W.A.* AND THOMAS, W., JR.**

THE IDENTIFICATION OF *Mycobacterium tuberculosis* in clinical specimens is one of the most tedious tasks confronting both the bacteriologist and pathologist. It is well known that the detection of acid-fast organisms is extremely difficult in conventionally stained smears especially when they contain only a few tubercle bacilli. Therefore, a method which is capable of detecting this pathogen more readily would certainly be of considerable value. Such a procedure has been discussed in the past but, although previously tried by us and many others, has not been widely accepted. The method referred to herein is the fluorescent technique employing the two dyes — auramine and rhodamine.

Our earlier experiences with the fluorescent method in 1954 were not too rewarding. The microscopic equipment used with this technique at that time was not nearly as adequate as the instruments which are readily available today. As a result, the procedure at that time required extremely careful supervision and proved to be impractical for the routine screening of acid-fast bacilli. However, during the past two years we have had the opportunity to re-evaluate the newer equipment, materials and procedures in both developmental research projects and routine acid-fast screening programs. The results have been very encouraging and the purpose of this report is to describe our experiences with the fluorescent method and to compare it with the conventional Ziehl-Neelsen procedure.

The experiments were designed to evaluate the usefulness of the fluorescent technique for the routine screening of all clinical specimens suspected to contain acid-fast bacilli. Auramine and rhodamine were used separately and in combination. The latter was found to be the most satisfactory procedure.

MATERIALS AND METHODS

All types of clinical specimens submitted for acid-fast studies during the two year period of this investigation are included in this report. The specimens included bronchial secretions, cerebrospinal fluid, exudates, gastric secretions, 24-50 hour sputum pools, catheterized urine and early morning or 24-hour voided specimens, as well as a great variety of material obtained

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*Department of Laboratories.
**Formerly with the Department of Laboratories. Presently at Bridgeport Connecticut General Hospital, Bridgeport, Connecticut.

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by all specialties in the departments of medicine and surgery. The materials were usually digested with either equal amounts of 15 or 23 per cent trisodium phosphate for an eighteen hour period at 37°C. They were subsequently neutralized and the sediments were used to inoculate three different types of media and for the preparation of smears for fluorescent and Ziehl-Neelsen staining. The media consisted of the following: (1) Lowenstein-Jensen, (2) Petragnani, and (3) ATS-American Trudeau Society. The cultures were routinely examined at weekly intervals for an 8-12 week period. If the culture media remained negative on material whose smears showed acid-fast organisms, the incubation was routinely continued for 6-12 months.

Staining Procedures

The procedures used at the beginning of these studies were varied considerably in an attempt to determine the optimum conditions for the detection of acid-fast bacilli in clinical specimens. Smears of typical and atypical *Mycobacterium*, as well as sediments of clinical materials were fixed by different methods; i.e. formalin or heat. The fluorescent dyes — auramine and rhodamine — were used either separately or in combination.

A. SOLUTIONS

I. Composition of fluorescent dyes.

i. *Auramine O* CI 41000*

Auramine	1.5 grams
Glycerol	37.5 ml
Phenol	5.0 ml
Distilled water	25.0 ml

ii. *Rhodamine B* CI 749**

Rhodamine	0.75 grams
Glycerol	37.5 ml
Phenol	5.0 ml
Distilled water	25.0 ml

iii. *Auramine and Rhodamine*

Auramine	1.5 grams
Rhodamine	0.75 grams
Glycerol	75.0 ml
Phenol	10.0 ml
Distilled water	50.0 ml

The solutions outlined above are almost saturated and there was no advantage in increasing the concentration. The solutions were clarified by filtration through glass wool. We have stored the stock solutions both in the refrigerator at 4°C and at room temperature for months without noticeable loss in staining characteristics.

II. Decolorizer.

Acid alcohol

0.5 per cent HCl in 70% ethanol

III. "Counterstain".

Potassium permanganate

0.5 grams in 100 ml of distilled water

*Produced by Allied Chemical certified for use in "fluorescent procedures"
**Obtained from Matheson, Coleman and Bell.

B. FIXATIVES

The smears and tissues were fixed in 10 per cent formalin solution. Heat fixation with a Micro-Slide Staining and Drying Bath* was performed on smears of pure cultures, as well as clinical specimens. Since the heat fixed specimens were comparable to the formalin fixed smears, the former was the method of choice for screening the routine smears prepared from clinical specimens.

C. STAINING METHOD

The smears were stained at either 37°C or 60°C for periods of 10-60 minutes. Adequate fluorescence was obtained with all positive smears. Therefore, we decided arbitrarily to choose the 15 minute period at 37°C for use in the routine laboratory.

D. DECOLORIZER AND "COUNTERSTAIN"

The stained smears were decolorized with O.5 per cent hydrochloric acid in ethanol for 2-3 minutes and then thoroughly rinsed. Time periods of 10 minutes with HCl were also very satisfactory. The smears were then flooded with 0.5 per cent potassium permanganate for 2-4 minutes, rinsed, dried and examined. The precise function of the "counterstain" is not completely understood but it renders tissue and its debris non-fluorescent, thus reducing the possibility of artifacts. Excessive exposure time of 4-10 minutes with the "counterstain" tends to produce a loss of brilliance. s.a. Kupor, Hoi

IV. Fluorescence Microscopy

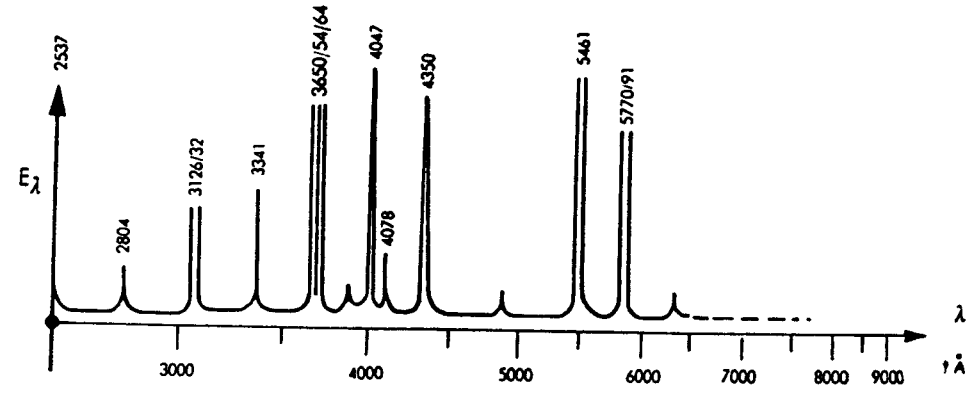
It is of considerable interest to mention that a great deal of the controversy in the past regarding fluorescence microscopy has been directly proportional to the difficulty in assembling adequate equipment. The latter was not commercially available as a "complete unit for fluorescence microscopy." Therefore, many investigators were forced to improvise with equipment which in most instances was not equal to the high standards of the apparatus available today.

Early in 1960, the decision was made to re-evaluate the fluorescent acid-fast technics because the Zeiss photomicroscope and its accompanying equipment appeared to meet all of the basic needs for fluorescence microscopy of this type. The major components supplied with this apparatus were as follows:

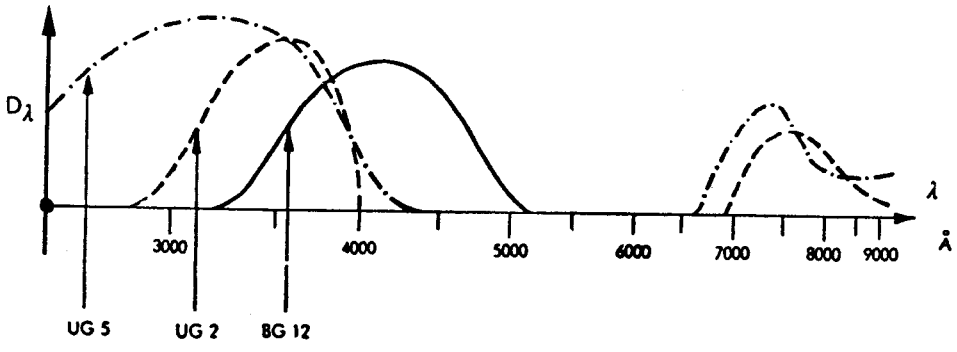
(i) The fluorescence lamp consists of an Osram HBO 200 mounted in a spherical lamp housing which can be tilted, swivelled and adjusted to the appropriate position. The mercury vapor lamp has a filament efficiency of 200 watts.

(ii) The illumination equipment with exciter filters supplies the need for producing the short-wave length rays so necessary to produce fluorescence with the dyes presently being used. The filters used with the photomicroscope consists of the primary BG 12/3 mm and BG 12/4 mm in conjunction with a heat protecting filter. The two barrier or secondary filters are inserted into

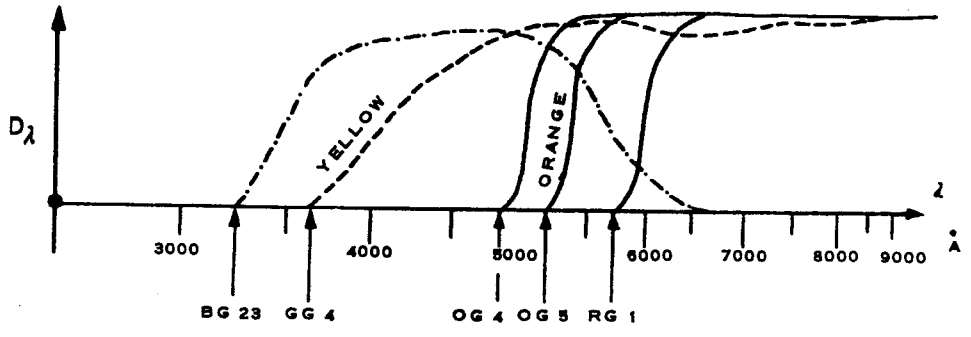
*This apparatus was produced by the Overland Electric Company, Cicero, Illinois.



Spectral emission of the maximum pressure mercury vapour lamp



Spectral transmission of the exciter filters



Spectral transmission of the barrier filters

Figure 1

Optical characteristics of the Osram 200 bulb, primary exciter filter and secondary barrier filters. (from Zeiss) Eλ=Energy output or emission; Dλ=Spectral transmission.

a slot in the tube-head and are designated as (1) yellow filter GG 4 and (2) orange filter OG 5. See figure 1 for the transmission curves* of these filters.

(iii) The Zeiss ultra condenser 1.1 with intermediate piece Z was used inter-

changeably with the bright field achromatic aplanatic condenser 1.4 Z. The former was used for fluorescence microscopy and the latter for the Ziehl-Neelsen technique. However, it should be emphasized that we found it more convenient to use a second microscope for bright field examinations rather than change the condensers.

(iv) Following is a list of the objectives and oculars used:

i Planapochromat 25/0.65 (N. A.)
ii Planachromat 40/0.65 (N. A.)
iii. Apochromat 40/1.0
oil immersion with iris diaphragm
iv. Apochromat 100/1.32 oil
immersion with iris diaphragm
v. Complan eye pieces KPL 10X
vi. Complan wide angle KPL 12.5X

(v) The photomicroscope is equipped with an automatic exposure device in the housing which will automatically bring about correct exposures with the assistance of its photoelectric cell. The device operates on the principle of integration of the brightness distribution in the central part of the image. Therefore, satisfactory results are not obtained if there are extreme irregularities in the brightness distribution, such as may occur with a clump of very brilliant acid-fast organisms.

Another Zeiss microscope identified as the GFL model has also been extensively used by us during the past year for the screening of clinical specimens suspected to contain acid-fast organisms. This instrument is essentially similar to the photomicroscope with a few exceptions which should be mentioned. The GFL model is less expensive than the photomicroscope and as a result has fewer elaborate features. It lacks the following: (1) Optovar (magnifying) attachment, (2) convenience of the rear field diaphragm attachment and (3) the automatic exposure equipment. However, the GFL has one important extra which consists of two revolving discs in the filter extension tube. This combination of filters permits greater flexibility in adjusting and varying the barrier filter positions and wave lengths (410-530 millimicrons).

V. Photomicrography

Two types of cameras were employed for recording the results by means of photomicrographs. Both the automatic camera device of the Zeiss photomicroscope and a manually operated Bausch and Lomb camera in conjunction with the Zeiss GFL model were used. Photomicrography of fluorescent tubercle bacilli presents some difficulty to the uninitiated. Determination of optimal exposure times required the employment of three procedures: (1) estimation of the brightness of the image, (2) measurement of the illumination intensity and (3) trial exposures. With the exceptionally wide range in brightness of fluorescent material, the first procedure can only accidentally lead to optimum results, the second requires unpopular computations, and the third is time-consuming. Thus the automatic control device (photoelectric

*The transmission curves were obtained from Zeiss bulletin G 40-215-e entitled, "Large Fluorescence Equipment".

cell) measures the luminous intensity of a portion of the image and translates this measured value into the correct exposure time. In addition, the photomicroscope is also equipped to advance the 35 mm film automatically.

Several types of color film have been tested for our color photography studies. The following is a list of those tested: Super Anscochrome 135 tungsten; Kodak Ektachrome E135, EF135, EH135 and EHB135; Kodachrome II daylight KR135; Kodachrome Type KA135; Kodacolor C135 daylight or flash.

The time exposures varied from 30 seconds to 8 minutes. In general, best results (see Plate 1) were obtained with Super Anscochrome 135 tungsten using the photomicroscope, or with Super Anscochrome cut film employing the Bausch and Lomb camera affixed to the Zeiss GFL microscope. The usual range of magnification was 400 to 1000X. The cells in Plate 1 are magnified approximately 400X.

Results

A comparison of various dye solutions using acid-fast containing preparations was undertaken. Auramine and rhodamine were used separately and in combination. Auramine-stained acid-fast organisms were yellow to orange in color whereas the rhodamine-stained cells were reddish. The artifacts seemed to be stained more prominently with auramine and were less likely to appear with a reddish tinge as did the acid-fast bacilli. The results obtained with the auramine-rhodamine combination compared more favorably with the rhodamine than with the auramine-stained smears.

The experimental and routine studies were usually performed with combinations of auramine and rhodamine. At the beginning of the experiments, both known positive and negative smears and tissues were employed as controls. The acid-fast bacilli were seen in various colors (see Plate 1) depending upon the type of barrier filter. The background color also varied according to the barrier filter, aperture of the objective (with diaphragm) etc. The bacilli were readily seen with the 25X objective which permitted a more rapid survey than the oil immersion objective.

Fluorescing artifacts present a problem to the uninitiated. However, if one examines several hundred positive preparations at various magnifications, the possibility of error is greatly reduced. After sufficient experience, especially with the oil immersion objective, the microscopist is able to recognize the typical characteristics such as beading, cording and the slender rod-like structures. The use of potassium permanganate as a "counterstain" greatly reduces the staining artifacts. Overexposure to potassium permanganate adversely affects the staining intensity of the acid-fast organisms if allowed to react for 10 minutes instead of the usual 2-3 minutes.

The next step in evaluating the reliability of the procedure was to determine the staining reaction of atypical acid-fast bacilli. Strains of photochromogens, scotochromogens and non-chromogens isolated in both our laboratory and at Herman Kiefer Hospital in Detroit were examined and found to retain the auramine-rhodamine stain.

A very important consideration at this phase of our experiments in addition to the comparative reliability of the two dyes was the question of possible fluorescent

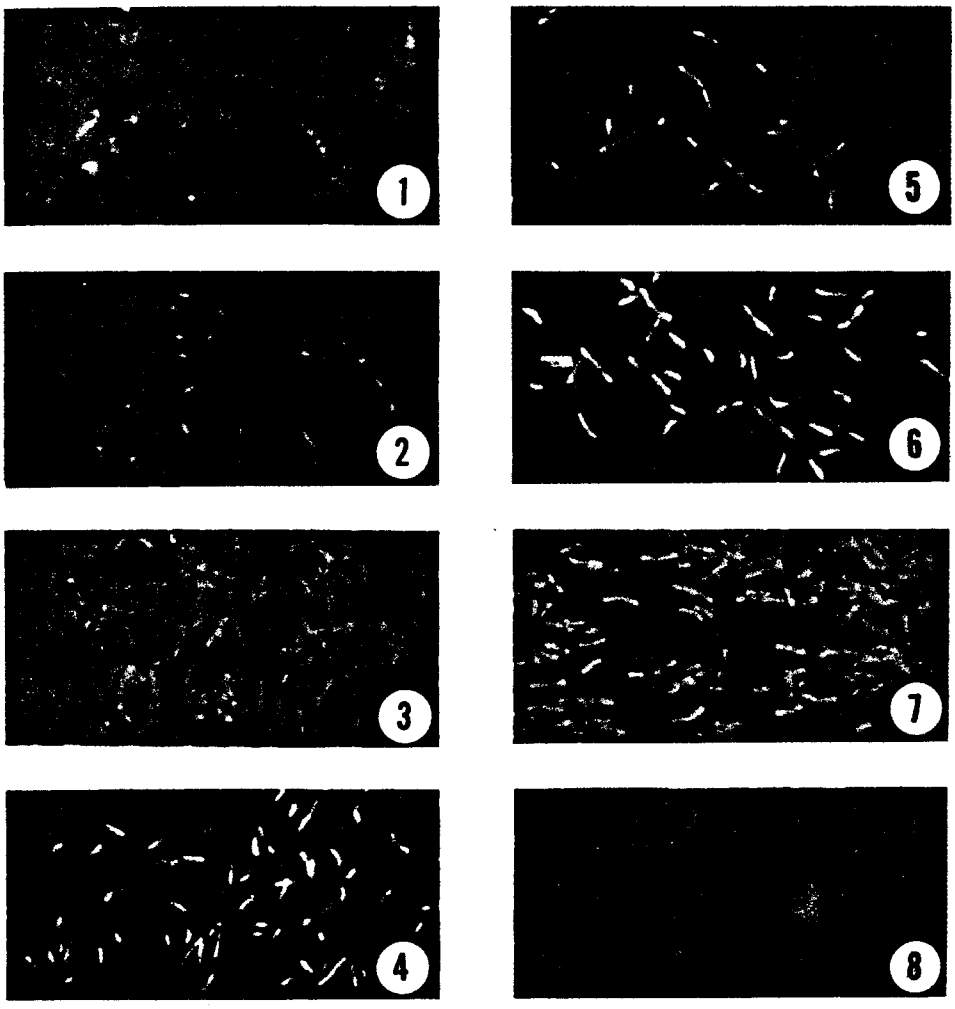


Plate I

Acid Fast Bacilli observed with the following barrier filters:

1. 410 mu	5. 470 mu
2. 440 mu	6. 500 mu
3. 440 mu	7. 500 mu
4. 470 mu	8. 530 mu

staining of organisms other than the acid-fast group. All the common vegetative strains *(Aerobacter, Bacillaceae, Corynebacteriaceae, Escherichia, Proteus, Pseudomonas,* staphylococci, and streptococci) did not retain the auramine-rhodamine stain. Thus, there is little likelihood of false positives due to the common oral and urogenital tract flora. Fluorescent-stained smears of *Actinomyces* species* obtained from the American Type Culture Collection were also negative.

*The species of *Actinomyces* were *A. bovis* strains #1829 and #1833 (human) and #B-99 (bovine); *A. israeli* #277 and #287 (human); *A. naeslundi* #279 (human.)

Another approach to the problem related to the identification of questionable artifacts was the possibility of counterstaining the auramine-rhodamine stained smear with the carbolfuchsin method. The results on both pure cultures and positive sediments showed that the majority of fluorescent acid-fast cells are poorly counterstained with carbolfuchsin, however, occasional chains were brilliantly stained. The reverse procedure which involved the counterstaining of Ziehl-Neelsen stained smears with auramine-rhodamine proved to be more successful with all preparations.

The results of the foregoing preliminary experiments were so encouraging that the authors decided to examine all specimens submitted to the bacteriology laboratory with both the conventional and the fluorescent technics. Two slides were stained with carbolfuchsin and one with the auramine-rhodamine combination for each specimen. The former slides were examined by the technologists assigned to the routine tuberculosis laboratory and the fluorescent-stained smears were examined by one of the authors. The positive results attributable to sampling should have been in favor of the conventional Ziehl-Neelsen procedure but in spite of this the fluorescent method was superior (see Table 1). It should be noted that there were four positive specimens with the Ziehl-Neelsen method as compared to seventeen in favor of the fluorescent technique. In addition, the latter procedure demonstrated the organisms within a 5-minute period of microscopic examination whereas the conventional method usually required a more prolonged search (10-20 minutes). Having examined 1000 specimens at this stage of our studies, it was obvious that the new method showed superior results, especially in the light of confirmation by the positive cultural findings (see Table 1).

Table I

Comparative efficacy of the methods for the detection of acid-fast bacilli

Type of Specimen	No. of Specimens	A-R+* and Z-N+** Culture +	A-R+ and Z-N- Culture +	A-R- and Z-N+ Culture +
sputum	585	43	13	4
urine	210	5	1	0
tissues	102	5	1	0
gastric	48	0	2	0

*A-R refers to the auramine-rhodamine procedure.
**Z-N identifies the Ziehl-Neelsen method.

It should be made clear that no single laboratory method for the diagnosis of tuberculosis is infallible (see Tables 2 and 3). The data in Table 2 shows a range of positive and negative findings observed by both microscopic and cultural procedures. This information stresses the important fact that both clinician and laboratory investigators should not depend solely on one method.

Discussion

The use of fluorescent dyes for the detection of acid-fast bacilli in clinical specimens was described by Hagemann[1] in 1937. The microscopic equipment used by Hagemann was not generally available at this relatively early date. The period between 1941 and and 1943 saw several investigators[2,3,4] give greater impetus to these

Table II

Cross-section of variations which occur with microscopic and cultural acid-fast procedures

Identifications of 1961 Specimens	A-R	Z-N	Culture
3-21-3	+	−	+
4-3-7	+	±*	+
4-6-3	±*	+	+
4-17-6	−	−	+
10-3-7	−	+	+
10-5-9	2+	1+	+
10-5-20	2+	1+	−
10-13-9**	1+	1+	+

*± refers to doubtful positive and requires further examination of specimen by staining techniques.
**both methods detected few atypical chains (*Mycobacterium* sp.) which were culturally identified as three chromogenic colonies.

Table III

Comparative efficacy of microscopic and cultural methods for detection of acid-fast bacilli in 3000 specimens*

Group	Microscopy A-R	Microscopy Z-N	Cultures	Per cent of Total
1	+	+	+	5.6
2	+	+	−	2.1
3	+	−	−	0.8
4	+	−	+	1.5
5	−	+	+	0.5
6	−	−	+	1.2
7	−	+	−	0.5
8	−	−	−	87.2

*12.8 per cent of the specimens were positive by one or more of the procedures.

studies by improving available equipment. Heretofore no comprehensive study had been reported of light sources, requisite excitation wave-bands, optical system, filters or stains, thus the procedure remained largely empirical until these authors published their very informative research.

However, fluorescence microscopy as a diagnostic aid in tuberculosis had yet gain universal support as discussed by Gray[5] in 1953. Lind[6] in 1949 reported that only two of twenty-four state laboratories in the United States considered the fluorescent method better than the conventional staining procedures. In 1954 the authors of this paper employed a modification of the equipment described by Matthaei[7] and the combination of dyes reported by Gray.[5] Our experiences at this time were in agreement with these investigators who up to this date had reported advantages but were quick to point out that the procedure required considerable experience with the equipment, technic and microscopic examination.

The fluorescent technique yielded slightly more positive smears than the conventional method in our experimental and routine studies. These results are in agreement with the more recent experiences of Needham[8], Kuper *et al*[9] and Braunstein *et al.*[10]. Needham stresses the findings that fifty five percent of specimens which are negative microscopically were proven to contain tubercle bacilli by culture and/or

animal inoculation. On this basis he rightfully concludes that the demonstration of acid-fast bacilli by culture and animal inoculation are superior to direct microscopic procedures. However, the fluorescent technique does yield a larger number of positives in smears and tissues than does the Ziehl-Neelsen method. This advantage in addition to the ease of examination (i.e. less eye strain for most microscopists) and the greater rapidity in detecting tubercle bacilli has led to the routine use of the fluorescent technique in our bacteriology laboratory.

Summary and Conclusions

Three thousand clinical specimens have been examined by the auramine-rhodamine procedure. The combination of dyes was superior to the use of separate dye solutions. The two models of Zeiss microscope proved extremely useful and under proper conditions the examination of smears and tissues by fluorescence microscopy involved less eye strain than with the conventional procedure.

Tubercle bacilli were detected with the 25X (low power) objective lens within seconds unless they occurred in extremely small numbers. Confirmation with the oil immersion objective was clear-cut since such typical morphological characteristics as beading and cording were outstanding.

The method is simple but care is essential in certain steps in adjusting the lamp, dark field condenser and the variable diaphragm of the oil immersion objective. The apparatus is comparatively expensive and the maintenance of the illuminator system is not cheap.

The method is recommended for the routine examination of potentially tuberculous clinical material.

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