SELECTED STUDIES OF THE FLUORESCENT STAINING OF ACID-FAST BACILLI IN BOVINE TISSUES WITH AURAMINE O

by

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A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
MASTER OF SCIENCE

Major Subject: Veterinary Pathology

Iowa State University
Of Science and Technology
Ames, Iowa

1966

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INTRODUCTION

One of the most tedious tasks confronting veterinary pathologists is the search for acid-fast bacilli in tissue sections suspected of containing tuberculous lesions. Whenever acid-fast bacilli are not demonstrated, the diagnosis of tuberculosis is presumptive. The problem is complicated because tissue changes found in tuberculosis may be produced by a number of other etiologic agents.

Each case of laboratory confirmed bovine tuberculosis encountered in slaughtering establishments and during routine tuberculosis eradication work must be traced to the herd of origin. Epidemiologic trace-back procedures are initiated upon the histopathologic demonstration of acid-fast bacilli within tissue changes typical of tuberculosis. Whenever acid-fast bacilli are not observed in the specimen during histopathologic examination, trace-back procedures are delayed until time-consuming mycobacteriologic studies are completed. Therefore, it is imperative to find the acid-fast bacilli if they are present.

Ideally, acid-fast bacilli should stain equally well in all instances. However, experience has shown that staining characteristics of mycobacteria in tissue sections are variable. Acid-fast bacilli are not always demonstrated by conventional methods even though they may be cultured from adjacent areas in the same lesion. This variable

staining (6) may be due to environmental effects related to the buildup of toxic metabolic products (21) and to anaerobiosis (47).

Fluorescent staining offers another means of demonstrating acid-fast bacilli. Pathologists in human medicine are particularly enthused by this procedure. It is accepted for staining sputum smears and is used on tissue sections from human patients. Fluorescent staining is effective in rapidly demonstrating acid-fast bacilli and is effectual in certain cases when conventional methods fail.

Application of the fluorescence microscopy method for the detection of acid-fast bacilli in bovine tissue sections has not been reported.

Because conventional staining procedures frequently fail to demonstrate acid-fast bacilli within typical tissue changes of tuberculosis, the need for a simple, sensitive, and effective staining method for detecting them is obvious. An adjunctive staining method for demonstrating such acid-fast bacilli is presented.

The purpose of this research was to investigate the fluorescent staining of acid-fast bacilli in bovine tissues stained with auramine O. This research will be restricted to selected groups of tissues from the tissue repository of the Pathology Unit, Diagnostic Services at the National Animal Disease Laboratory. The procedure, results, discussion, and conclusions of this research follow.

LITERATURE REVIEW

The spontaneous fluorescence of some substances was observed by Kohler (32) in 1904 while experimenting with the darkfield microscope and ultraviolet light.

Arloing, Policard, and Langeron (1) in 1925 noticed the spontaneous fluorescence of human tubercle bacilli was gray; a bovine strain of tubercle bacilli was violet.

Haitinger and Hamperl (24) undertook an extensive and a systematic investigation of the various fluorochromes, including auramine, that were in rather common usage in 1933.

In 1937 Hagemann (22) observed the fluorescence of tubercle and leprosy bacilli after staining with berberine sulfate in phenol and decolorizing with acid-alcohol solution. Later, Hagemann (23) found auramine superior to berberine sulfate for the fluorescent staining of tubercle bacilli in smears. He felt this procedure shortened the time for finding acid-fast bacilli and resulted in an increased number of positive cases of tuberculosis.

Herrmann (26) in 1938 discussed the advantages of the fluorescent method and concluded more positive cases of tuberculosis were found with the fluorescent technique than with the Ziehl-Neelsen stain.

Keller (30) in 1938 was the first to replace ultraviolet light by blue light as the source to excite fluorescence of auramine O.

Kuster (33) and Clauberg (10) in 1939 reported on satisfactory results using the fluorescent technique for detecting tubercle and leprosy bacilli in smear preparations.

Didion (14) in 1939 compared four techniques for demonstrating acid-fast bacilli. These techniques were Ziehl-Neelsen staining, fluorescent staining, bacteriologic culture, and animal inoculation. The results of his comparison on a percentage rating were as follows: animal inoculation was 100 percent, bacteriologic culture was 92 percent, fluorescent staining was 87.2 percent, and Ziehl-Neelsen staining was 82.2 percent.

Bachmann and Finke (3) confirmed the advantages of Hagemann's procedure of fluorescent staining. They also used this procedure to stain acid-fast bacilli in tissue sections. Finke (16) discussed the merits of acid-fast staining procedures using various fluorochromes in 1940. He also investigated the auramine staining of several types of acid-fast bacilli.

Rihl (46) and Oscarsson (41) elaborated on the fluorescence of acid-fast bacilli in 1940 and 1941, respectively. Oscarsson noted positive cases using auramine which were not confirmed by bacteriologic culture or animal inoculation.

Tanner (51) and Thompson (53) each wrote favorably in independent publications of their experiences with fluorescence microscopy utilizing auramine in detecting acid-fast

bacilli in tissue sections at the Mayo Clinic. Tanner found fluorescent staining of advantage in the study of the relationship of the tissue background and the acid-fast bacilli. In consideration of Finke's criticism of the Hagemann procedure utilizing auramine O as being unsuitable for tissue sections because of the background fluorescence, Tanner (51) utilized a modification of Herrmann's technique on his tissue cases. One hundred and thirty paraffin blocks were examined by the fluorescent procedure and the Ziehl-Neelsen technique. Auramine staining resulted in eight more positive cases of tuberculosis; of the Ziehl-Neelsen positive cases, three were negative to auramine. Tanner (50) compared fields in tissue sections stained with auramine and then by carbol-fuchsin. More acid-fast bacilli were seen by fluorescence microscopy, and only rarely did carbol-fuchsin show any which had not been shown by auramine.

Thompson (52) investigated a series of cases showing conflicting results with auramine and carbol-fuchsin. Of 55 cases examined, 50 were positive to auramine staining alone and five to carbol-fuchsin staining alone. Eighty percent of the cases in each of these groups were confirmed by other means.

Bogen (5) compared 1,000 duplicate smears stained in parallel by the Ziehl-Neelsen and fluorescent methods.

The efficiency of the fluorescent procedure was more than 20 percent better than the Ziehl-Neelsen procedure. Bacteriologic cultures confirmed the specificity of the fluorescent staining.

Lind and Shaughnessy (36) concluded the fluorescent technique for the examination of tubercle bacilli was more sensitive than the Ziehl-Neelsen staining method. The positive results were increased by one percent and apparently the time required to examine each case was reduced by one-fourth to one-third.

Richards, Kline, and Leach (44) and Richards and Miller (45) in 1941 compared fields in smears stained by auramine and carbol-fuchsin. More organisms were stained by auramine O, but occasional bacilli which had not been stained by auramine were stained by carbol-fuchsin.

Richards (43) had shown that tubercle bacilli are stained with auramine O by combining with mycolic acid; he stated that acid-fast bacilli are stained similarly with carbol-fuchsin. Auramine was retained much more intensely by mycolic acid.

Graham (20) in 1943 showed that the effective wave lengths for the fluorescence of auramine extend well above the ultraviolet range. This confirmed Keller's technique of replacing ultraviolet light by blue light as a light source for fluorescence microscopy.

Dienst (15) found auramine and carbol-fuchsin equally effective in known cases, but he preferred the latter because the cytological detail was good.

Van Dyke (54) studied 151 smears containing acid-fast bacilli. He reported all were positive to auramine and all but one were verified by bacteriologic culture.

Bekker and Tasman (4) in 1941 published that acid-fastness and fluorescence of Mycobacterium tuberculosis were removed by chloroform. They suggested that both properties were associated with the lipoidal fraction of the bacillary organisms.

Hauduroy and Posternak (25) in 1942 gave a review of the microscopic features and related microscopy of the tubercle bacillus in fluorescent light involving Hagemann's fluorescent staining procedure.

Freiman and Mokotoff (17) wrote a treatise about the comparison of the findings of many cases utilizing the Cooper modification of the Ziehl-Neelsen technique and the fluorescent method. A significant increase in the number of positive specimens found by fluorescence was obtained in gastric contents. Differences observed in the examination of untreated and concentrated sputum specimens were insignificant and in the latter case were less than the differences obtained when a series of duplicate smears were examined by the Ziehl-Neelsen modification alone.

Crossman and Lowenstein (12) in 1943 described a staining method using carbol-auramine, acid-alcohol decolorization, and methylene blue counterstain in tissue sections.

Lempert (35) developed a modified fluorescent method in 1944 that used variable-colored filters to highlight the auramine-stained bacilli.

Lee (34) evaluated several procedures of staining tubercle bacilli using auramine in fluorescence microscopy. After extensive trials with various acid-alcohol solutions, he chose one of 24 percent sulfuric acid in 70 percent ethanol.

Petrini (42) compared auramine and Ziehl-Neelsen techniques. He found out of 227 cases, 151 were positive for acid-fast bacilli with the auramine technique, whereas 136 cases were positive for tubercle bacilli with the Ziehl-Neelsen technique.

Hughes (27) showed that the exciting wave lengths for auramine did actually include the blue portion of the visible spectrum. Moreover, he states that it is no problem to create blue light of sufficient strength using proper filter systems and he says that blue light is more easily transmitted through normal optical glass.

Clegg and Foster-Carter (11) in their 1946 dissertation compared the reliability and convenience of the fluorescent procedure with the Ziehl-Neelsen technique. They concluded

that the fluorescent method was just as accurate, more sensitive, and faster than the Ziehl-Neelsen technique.

Briggs and Jennison (7) in 1947 modified the fluorescent method by using a more specific filter combination to enhance better contrast. In comparing a new method of acid-fast staining developed by Jennison with both auramine and Ziehl-Neelsen staining, they concluded that the fluorescent technique was equal to the new method. However, they stated that both methods were greatly superior to the orthodox Ziehl-Neelsen stain. It is interesting to note that in using the auramine method, permanganate counterstaining was used (Herrmann procedure); they made mention of the fact that potassium permanganate tends to mask the less brilliant organisms.

A paper by Von Haebler and Murray (55) in 1954 concluded fluorescent staining has many favorable advantages in its ability to detect acid-fast bacilli.

De Gommier (13) published his findings on the detection of acid-fast bacilli using auramine. He favored the fluorescent technique over Ziehl-Neelsen staining.

Clark and Hench (9) eluded to the excellent quenching qualities of ferric chloride with regard to the extraneous fluorescence of some fluorochromes in 1962.

Gilkerson and Kanner (19) and Kanner, Gilkerson, and Hogue (28) in 1963 found auramine to be more sensitive and

not less specific than other routine acid-fast staining procedures.

Koch and Cote (31) reported fluorescence microscopy incorporating auramine was far more superior to the time-honored Ziehl-Neelsen stain for the demonstration of acid-fast bacilli in tissue sections in 1965. Their research involved only human tissues.

PROCEDURE

Since the main objective of this study involved the fluorescent staining of selected groups of bovine tissue specimens with auramine O to detect acid-fast bacilli in tissue sections, no attempt was made to correlate the results with the clinical findings in individual or herd cases. The primary aim was to develop and establish fluorescent staining with auramine O as a routine method of detecting acid-fast bacilli in bovine tissue sections.

Material Specifications for Selected Studies

The material for this study consisted of formalin-fixed specimens of bovine tissues taken from the tissue repository of the Pathology Unit, Diagnostic Services at the National Animal Disease Laboratory. These tissue specimens were restricted to those that were received during the calendar years of 1964 and 1965.

These bovine tissue specimens originated from either of two sources; first, from cattle that were reactors or members of quarantined herds in the bovine tuberculosis eradication program of the Animal Health Division, and second, from bovine carcasses that contained lesions suspected of being tuberculous upon examination by the Meat Inspection Division. The bulk of the submitted bovine tissue specimens were lymph node tissue; however, liver, spleen,

lung, kidney, heart, mammary gland, skeletal muscle, adrenal gland, and salivary gland tissues were received in some instances. Skin tissue lesions were not considered in this study.

The design of this study was constructed so that one could determine from hematoxylin-eosin stained slides if a situation was presented to classify the respective cases into either granulomatous disease conditions, non-granulomatous disease conditions, or no microscopic lesions. Harris's alum hematoxylin and ethyl-eosin combination stain (2) was considered the superior stain available for demonstrating cytological detail in this study. This hematoxylin-eosin stain is used routinely in Diagnostic Services at the National Animal Disease Laboratory.

All tissue sections were stained by the new-fuchsin technique of acid-fast staining to detect acid-fast bacilli (56). It has been determined through long experience that the new-fuchsin technique of acid-fast staining is the best tissue stain available for detecting acid-fast bacilli in tissue specimens in Diagnostic Services at the National Animal Disease Laboratory. This was done to further classify the respective cases by the presence or absence of acid-fast bacilli into either tuberculosis or not tuberculosis in conjunction with the hematoxylin-eosin staining results.

Group I

Cases in the first selected group were all granulomatous disease conditions. Cases in this group were observed to contain at least two acid-fast bacilli per lesion of a granulomatous disease condition. This group was composed of all the positive tuberculous specimens of bovine tissue origin from January 1, 1964 to July 1, 1965.

Group II

Cases included in the second selected group were of the category whereby no microscopic lesions of any form or type were evident. These cases were noted to have no evident acid-fast bacilli anywhere in the apparently normal tissue. This selected group was made up of all the negative specimens of bovine tissue origin in which no microscopic lesions of any form or type were observed from January 1, 1965 to July 1, 1965.

Group III

All those cases in the third selected group were of granulomatous and non-granulomatous disease conditions. This group was examined and found not to contain evident acid-fast bacilli in these granulomatous or non-granulomatous lesions.

This group was composed of all the granulomatous disease conditions other than tuberculosis as well as non-granulomatous

disease conditions. Among the granulomatous disease conditions were actinomycosis, actinobacillosis, corynebacterial abscesses, migrating parasitic lesions, coccidioidomycosis, streptococcal abscesses, botryomycosis, phycomycosis, lipogranulomatosis, and foreign body granulomatous cases. The non-granulomatous disease conditions consisted of the multiplicity of neoplasms, septicemias, eosinophilic myositis, bronchopneumonitis, bacterial abscesses, congenital retention cysts, and other inflammatory disease conditions of a non-granulomatous disease nature.

This group is dated in the period of January 1, 1965 to July 1, 1965.

Group IV

Cases in the fourth selected group were either granulomatous disease conditions or no microscopic lesions. These cases were seen to have at least two or more acid-fast bacilli per lesion that was compatible with a granulomatous disease condition or no acid-fast bacilli that were apparent in normal tissue. This group was a group composed of tissue specimens from two laboratory animals (bovine) that were infected with Mycobacterium bovis and tissue specimens from a negative control animal (bovine) that was known to be free from tuberculosis. Two adult bovine animals were experimentally infected with Mycobacterium

bovis and held in isolation approximately two years. At necropsy, specimens containing tuberculous lesions were collected from these animals. Negative specimens were collected from one animal procured from a herd known to be free of bovine tuberculosis. This animal was negative on at least two tests using mammalian tuberculin injected intradermally while in detention for at least 15 months at the National Animal Disease Laboratory. Thorough post-mortem examinations were conducted and no lesions were found.

Methods Incorporated in Selected Studies

Histopathologic preparation

The bovine tissue specimens were identified by suitable record-keeping procedures. The specimens were fixed in ten percent buffered formalin.

Tissue specimens were adequately trimmed and blocked into sizes sufficient to be processed through Lipshaw tissue capsules.[1] The specimens were demineralized, if necessary, in five percent nitric acid solution for a minimum of two and one-half hours under 20 pounds negative pressure in a modified household pressure cooker. More time was allowed if the tissue specimens were excessively

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[1]Lipshaw Manufacturing Company, 7446 Central Avenue, Detroit, Michigan.

mineralized. The tissue specimens were neutralized in five percent ammonium hydroxide for one-half hour under 20 pounds negative pressure.

The tissue specimens were processed through an Autotechnicon[1] using Technicon dehydrant, formula S-29, and Technicon clearing agent, formula C-650, overnight for 14 hours, embedded in Tissuemat[2] within suitable mold forms that morning, sectioned at six micra with a Leitz rotary microtome, large Minot model No. 1212,[3] and mounted in triplicate in commercial egg albumin medium, Mayer's fixative.[4]

Fluorochroming Procedure

The procedure used in this study involved fluorochroming with auramine O by the following specific steps:

1. Deparaffinize through three changes of xylene, three changes of absolute ethanol, 95 percent ethanol, and 80 percent ethanol
2. Stain in auramine O for ten minutes

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[1]Technicon Company, Saw Mill River Road, Chauncey, New York.

[2]Fisher Scientific Company, 203 Fisher Building, 711 Forbes Street, Pittsburgh, Pennsylvania.

[3]E. Leitz, Incorporated, 468 Park Avenue, So., New York, New York.

[4]Fisher Scientific Company, 203 Fisher Building, 711 Forbes Street, Pittsburgh, Pennsylvania.

3. Decolorize in aqueous 20 percent sulphuric acid until decolorized

4. Differentiate in 80 percent ethanol for one minute

5. Quench in ten percent ferric chloride solution for five minutes

6. Immerse in 80 percent ethanol quite quickly

7. Dip in 95 percent ethanol for one minute

8. Dry in slide dryer, Lipshaw electric laboratory dryer,[1] for 15 minutes

9. Mount in non-fluorescent medium under special coverslip, Corning cover glass, No. 1.5 thickness.[2]

The auramine O stain was made up by the following formula:

Auramine O[3]	3.0 gm.
Glycerol	70.0 ml.
Phenol (liquefied)	32.0 ml.
Distilled water	900.0 ml.

Filter through cotton in a glass funnel several times.

The aqueous sulphuric acid solution was made up by the following formula:

Sulphuric acid, concentrated	200.0 ml.
Distilled water	800.0 ml.

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[1]Lipshaw Manufacturing Company, 7446 Central Avenue, Detroit, Michigan.

[2]Corning Glass Works, 1946 Crystal Street, Corning, New York.

[3]Matheson, Coleman, and Bell, Division of Matheson Company, Incorporated, 333 Patterson Plank Road, East Rutherford, New Jersey.

Add the acid very slowly to the water over the span of one hour in a protected area.

Ferric chloride for quenching extraneous fluorescence was formulated as follows:

Ferric chloride ($FeCl_3 \cdot 6\ H_2O$)	10.0 gm.
Distilled water	100.0 ml.

Dissolve the ferric chloride in the water and let the solution stand at room temperature for three days before using the solution.

The non-fluorescent mounting medium consisted of the following ingredients:

Styron 666[1] (K 27, clear, No. 71 granulation)	10.0 gm.
Dibutyl phthalate	5.0 ml.
Xylene	35.0 ml.

The styron must be thoroughly dried in a drying oven at 75 degrees Centigrade for two hours before use. The mixture is allowed to stand overnight for 12 to 14 hours. More xylene may be added if the consistency is too thick to allow for efficient mounting of the special coverslips.

Some variable factors, both physical and chemical, that affect the fluorescence of auramine O were carefully investigated. The approximate percentage solubility at 15 degrees Centigrade in water was one percent, in ethyl alcohol was four percent, in cellosolve was 1.25 percent, in ethylene glycol was 1.75 percent, and in xylol was 0.005 percent. It was readily dissolved in phenol. The ideal pH of the

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[1]Dow Chemical Company, 1000 Main Street, Midland, Michigan.

staining medium was found to be in the range of 6.2 to 8.4. If the solutions were heated above 38 to 40 degrees Centigrade, decomposition took place.

The absorption characteristics of auramine O were studied by means of spectrofluorophotography using an Osram HBO 200 mercury lamp, the mercury lamp used in the Leitz Ortholux research microscope adapted for fluorescent microscopy, and a Turner spectrofluorophotometer.[1] Figure 1 illustrates the spectrofluorophotogram of a 0.3 percent auramine O in a solution of glycerol, phenol, and water.

Fluorescence Microscopy

The fluorescence microscopy system used in the study was composed of a basic darkfield Leitz Ortholux research microscope;[2] however, the system was modified for fluorescent microscopy. The fluorescent light pathway and main components of the Leitz Ortholux research microscope[2] adapted for fluorescent microscopy in this study is shown in Figure 2.

A darkfield condenser adaptable to this system was utilized. This darkfield condenser was a Leitz darkfield

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[1]G. K. Turner Associates, 2524-26 Pulgas Avenue, Palo Alto, California.

[2]E. Leitz, Incorporated, 468 Park Avenue, So., New York, New York.

Figure 1. Fluorescent spectrophotogram of auramine O in the range of radiation corresponding to the electronic excitation of the Osram HBO 200 mercury lamp used in the Leitz Ortholux research microscope.

0.3 percent auramine O in a solution of glycerol, phenol, and water.

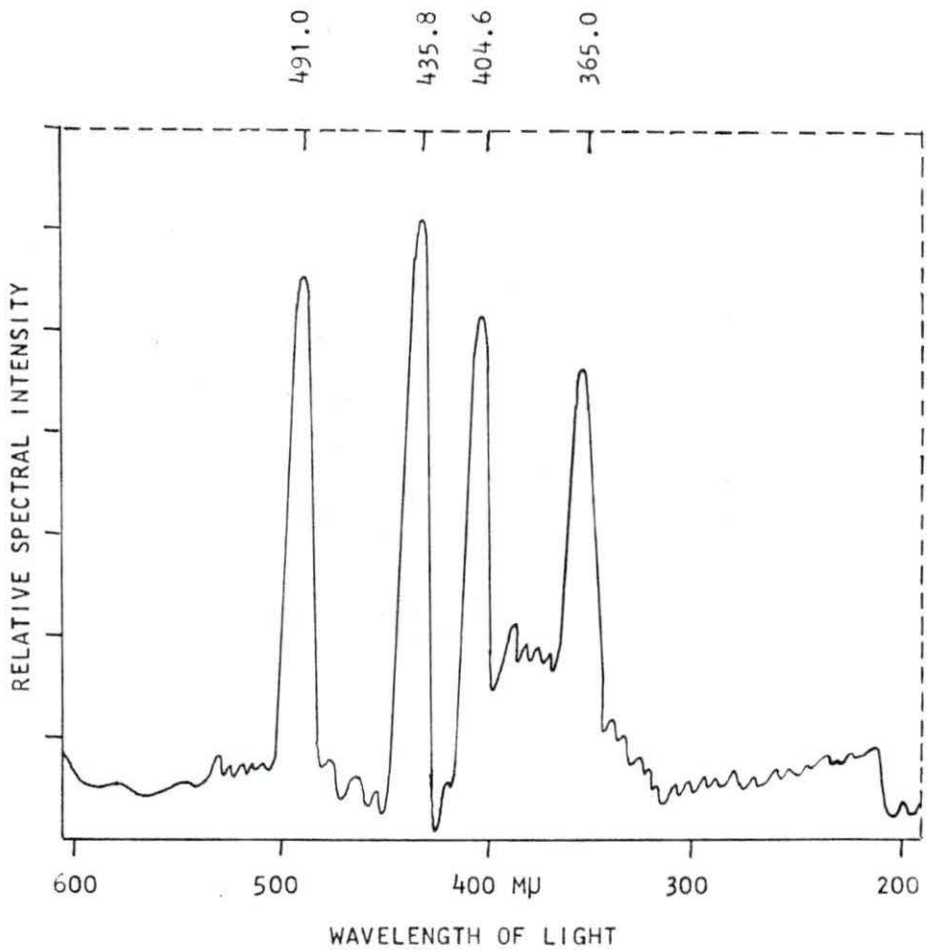
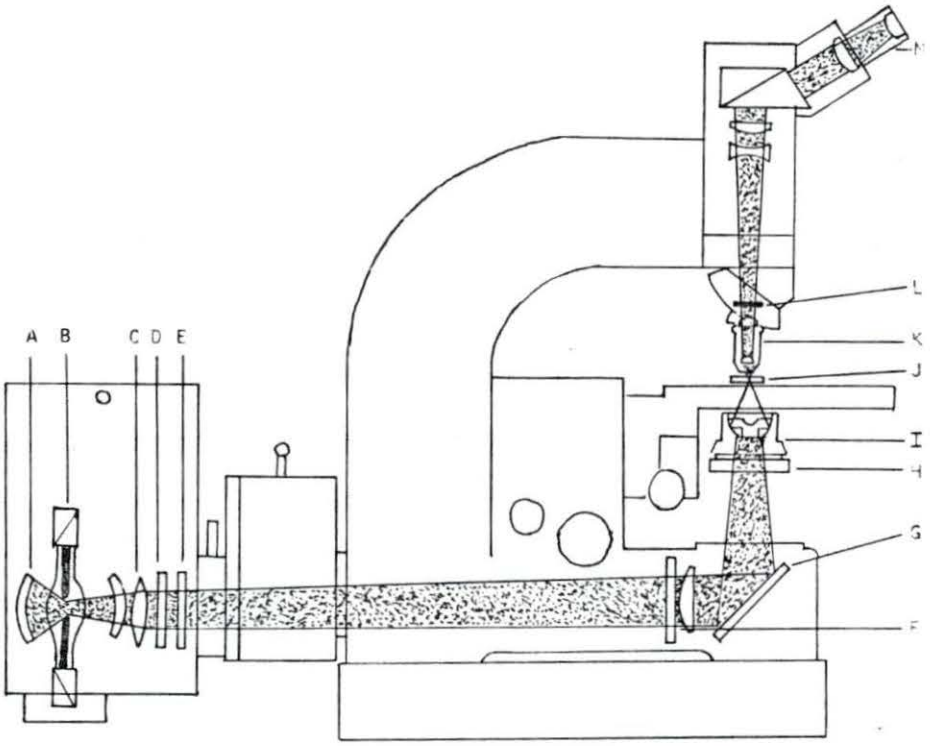

491.0
435.8
404.6
365.0
RELATIVE SPECTRAL INTENSITY
600
500
400 Mμ
300
200
WAVELENGTH OF LIGHT

Figure 2. Light pathway and main components of the Leitz Ortholux research microscope adapted for fluorescence microscopy.

A. Reflector
B. Mercury lamp (Osram HBO 200)
C. Collector lenses
D. Heat filter (Jena type BG-38)
E. Primary filter (Jena type BG-12)
F. Field diaphragm
G. Surface reflecting mirror
H. Aperture diaphragm and condenser
I. Darkfield condenser
J. Slide and specimen
K. Microscope objective lens
L. Secondary filter (Jena type OG-1)
M. Microscope ocular lens


A
B
C
D
E
L
K
J
I
H
G
F

condenser, dry type,[1] No. 85, with a numerical aperture of 0.80. The fluorescent light pathway and components of the darkfield condenser used with the Leitz Ortholux research microscope is depicted in Figure 3.

A light source suitable for fluorescence microscopy was used in this study. The light source was an Osram high-pressure, mercury vapor, 200 watt (HBO 200 w.) lamp contained within a Leitz lamp housing system.[1]

Spectrophotographic studies were carried out. The height and width of the arc are 2.5 and 1.3 millimeters, respectively, and the emission curve, shown in Figure 4, has main peaks at 365 and 435 millimicrons (range 280 to 600 millimicrons).

The light source generates much heat as well as light and it is, therefore, enclosed in a specially designed air-cooled housing.

A special starter unit provided transient high tension of about 15,000 volts to strike the arc, followed by a continuous low tension of about 60 volts to maintain the arc.

The rated life of an Osram HBO 200 w. lamp was averaged at about 220 hours.

The primary filter used was the OG 12 exciter filter of the Jena type.[1] This filter was situated in front of the

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[1]E. Leitz, Incorporated, 468 Park Avenue, So., New York, New York.

Figure 3. Light pathway and diagram of the darkfield condenser used in the Leitz Ortholux research microscope adapted for fluorescence microscopy (letters correspond to those of Figure 2).

I. Darkfield condenser
K. Microscope objective lens

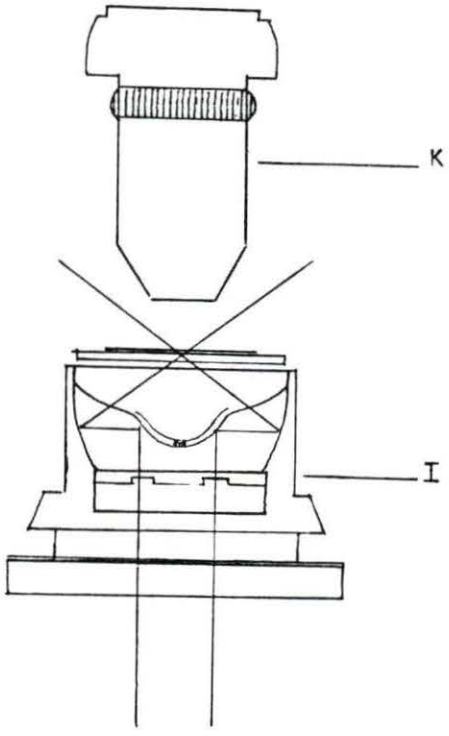
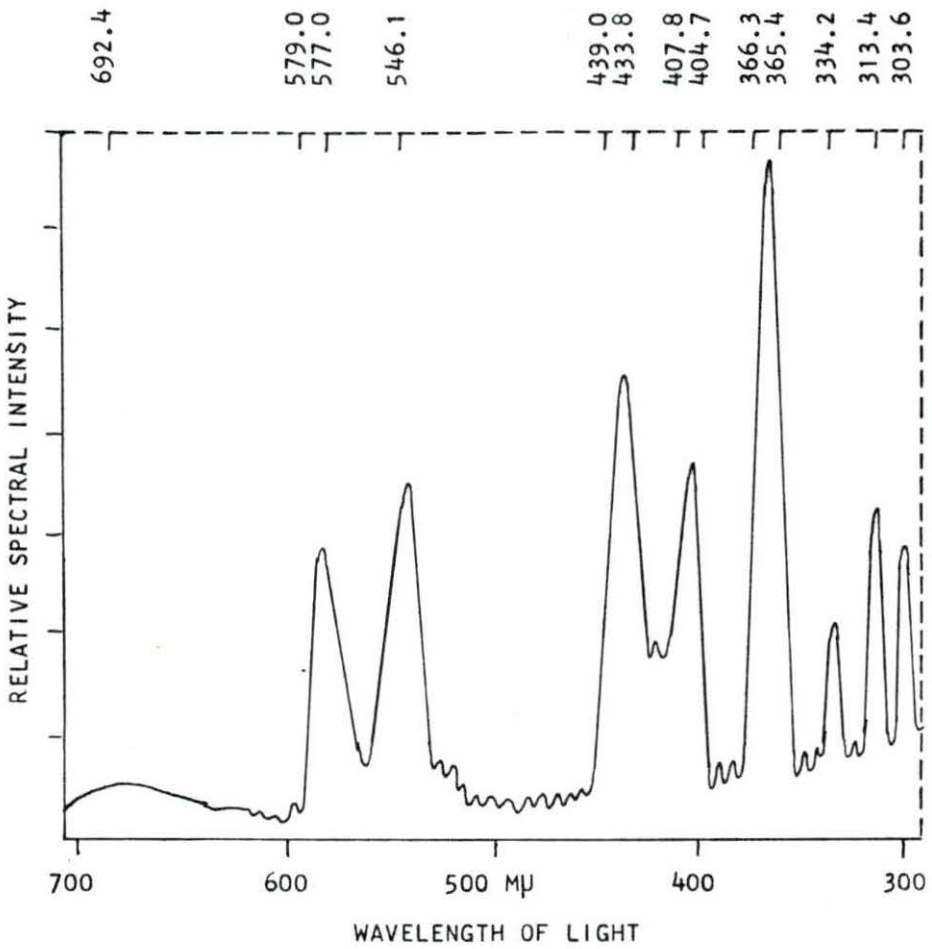

K
I

Figure 4. Spectral characteristics of the Osram HBO 200 mercury lamp as determined by spectrophotometric means.

Note that the spectral intensity peaks correspond almost exactly with the spectral peaks of auramine O. This is an almost absolute requirement of diagnostic fluorescence microscopy.


692.4
579.0
577.0
546.1
439.0
433.8
407.8
404.7
366.3
365.4
334.2
313.4
303.6
RELATIVE SPECTRAL INTENSITY
700
600
500 mμ
400
300
WAVELENGTH OF LIGHT

lamp housing behind a BG 38 heat filter. The transmission spectrophotogram of this primary filter is seen in Figure 5. This primary filter was used in the Leitz Ortholux research microscope adapted for fluorescence microscopy.

The secondary filter incorporated in this study was the OG 1 barrier filter of the Jena type.[1] This filter was located in the body of the Leitz Ortholux research microscope above the nosepiece. Figure 6 illustrates the transmission spectrophotogram of this secondary filter.

Suitable ocular and objective lenses for fluorescence microscopy with auramine O were incorporated into this study.

The ocular lenses were Leitz widefield type[1] of the magnification of six diameters with soft rubber eye-cups.

The objective lenses that were used were a Leitz apochromatic dry lens[1] of the magnification of 12.5 diameters, a Leitz apochromatic dry lens[1] of the magnification of 25 diameters, a Leitz fluorite dry lens[1] of the magnification of 40 diameters, a Leitz fluorite oil lens[1] of the magnification of 54 diameters, and a Leitz fluorite oil lens[1] of the magnification of 70 diameters. The corresponding numerical aperture figures for these lenses are 0.30, 0.65, 0.85, 0.95, and 1.30, respectively.

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[1]E. Leitz, Incorporated, 468 Park Avenue, So., New York, New York.

Figure 5. Transmission spectrophotogram of the primary filter (Jena type BG-12) used in the Leitz Ortholux research microscope adapted for fluorescence microscopy.

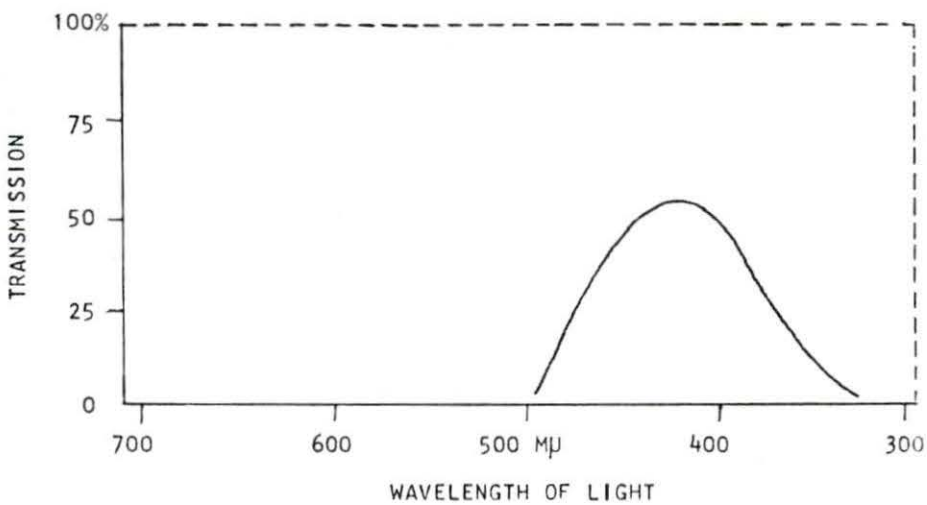
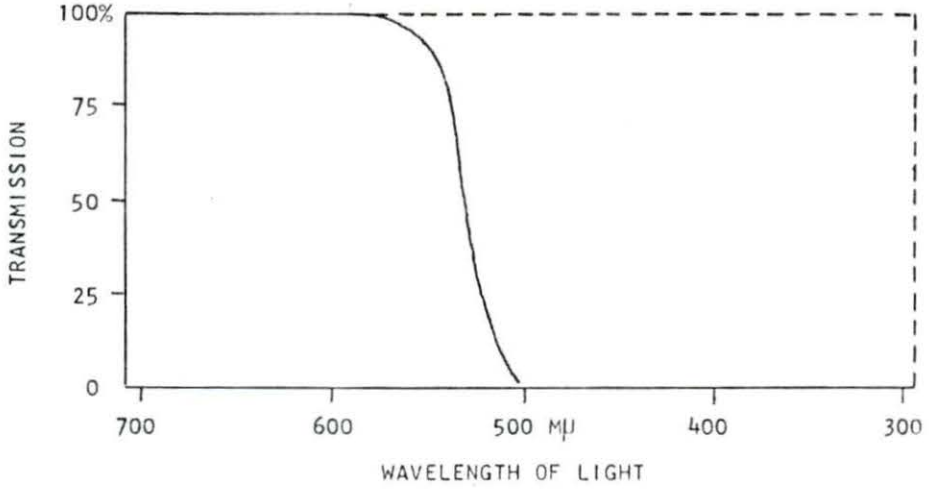

100%
75
50
25
0
TRANSMISSION
700
600
500 mμ
400
300
WAVELENGTH OF LIGHT

Figure 6. Transmission spectrophotogram of the secondary filter (Jena type OG-1) used in the Leitz Ortholux research microscope adapted for fluorescence microscopy.


100%
75
50
25
0
TRANSMISSION
700
600
500 MU
400
300
WAVELENGTH OF LIGHT

Fluorescent Photomicrography

The photomicrographic equipment that was used in this study was a Leitz Orthomat,[1] a fully automatic microscope camera. This camera system fits into the top of the microscope body by means of an adaptor. This camera is a 35 millimeter set-up for use in fluorescence, darkfield microscopy. It is possible to concentrate solely on the microscopic observation while recording photographically any important feature of the examination by pressing a single button. It is suitable for the extremely long time required for exposures of fluorescent objects, such as acid-fast bacilli.

The photographic film that was utilized in this study was of two types. Colored photomicrographs were taken with Kodak High-Speed Ektachrome (daylight)[2] with an ASA of 160 and Kodak High-Speed Ektachrome (artificial light)[2] with an ASA of 125.

The better colored photomicrographs were consistently taken with Kodak High-Speed Ektachrome (daylight).

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[1]E. Leitz, Incorporated, 468 Park Avenue, So., New York, New York.

[2]Eastman Kodak Company, 343 State Street, Rochester, New York.

Mycobacteriologic Techniques

All tissue specimens, if available, were submitted in either borax or chloramine T for mycobacteriology. The mycobacteriologic techniques utilized in this study were primarily those of W. L. Mallmann and associates (37, 38, 39). Specific typing procedures for detecting Mycobacterium bovis, Mycobacterium avium, and the atypical mycobacteria were utilized in the interest of saving time and expense (18, 29). These typing procedures eliminated the requirement for lengthy and costly laboratory animal inoculation studies.

RESULTS

This study was conducted on 1,737 cases divided into four groups. Group I consisted of 1,111 cases classified histopathologically as compatible with tuberculosis. Group II was composed of 327 cases that were histopathologically negative or non-lesion cases. Group III consisted of 296 cases containing lesions histopathologically indicative of both granulomatous and non-granulomatous disease conditions including some suggestive tuberculosis. Group IV consisted of two experimentally-produced cases of Mycobacterium bovis infection and one negative control case.

The results of this study were correlated in all instances between the fluorescent technique incorporating auramine O and the mycobacteriologic results whenever such were available.

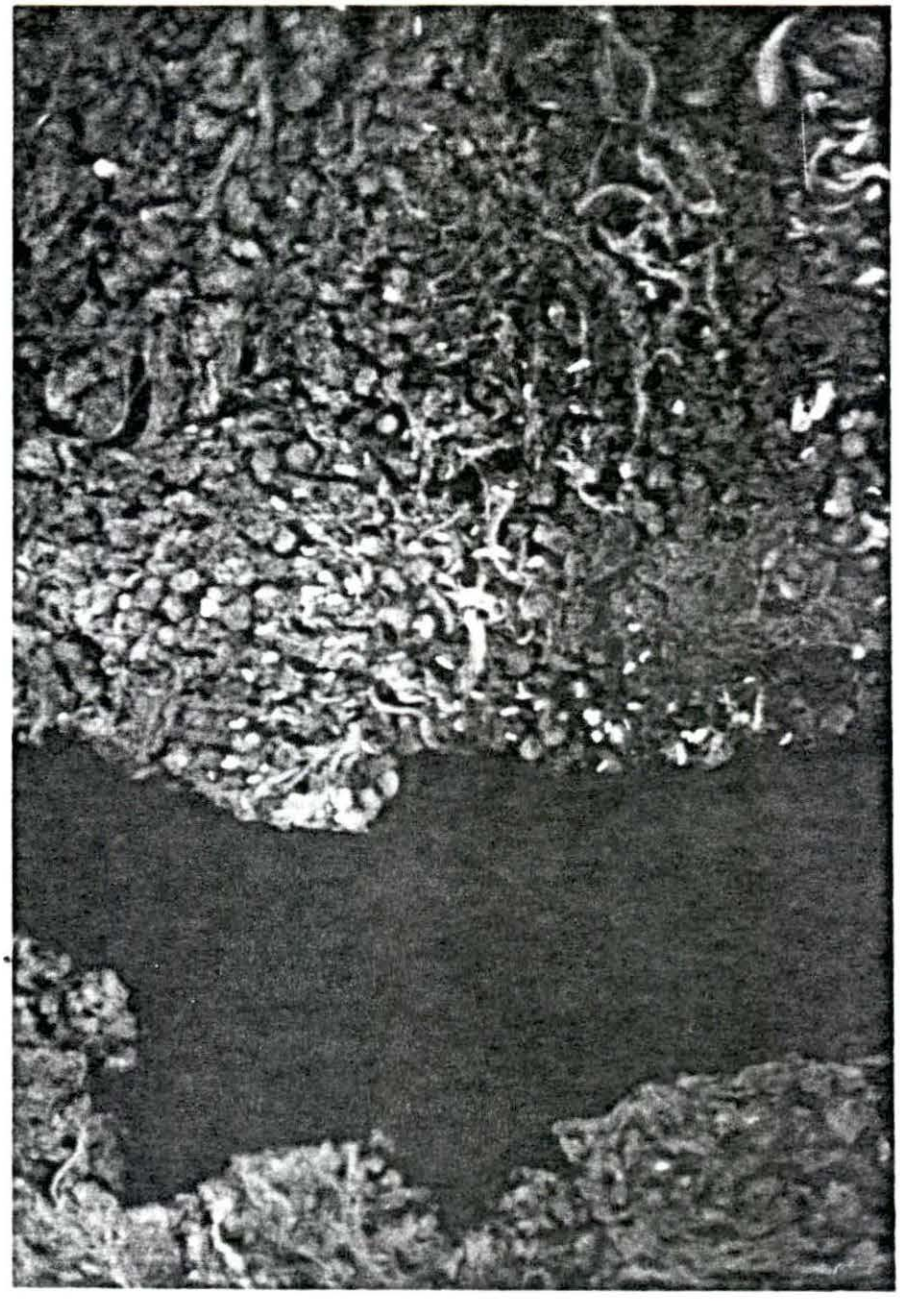
Group I

All of the 1,111 tuberculous cases in Group I were positive upon fluorescent acid-fast staining with auramine O. Mycobacteriologic techniques resulted in the isolation of Mycobacterium bovis in 452 cases.

Figure 7 shows a number of fluorescing acid-fast bacilli stained with auramine O in epithelioid cells of a lesion of tuberculosis. This lesion was present in a

Figure 7. Fluorescent photomicrograph of acid-fast bacilli in epithelioid cells of a lesion of tuberculosis in lymph node tissue from a bovine reactor animal. Mycobacterium bovis was isolated from this lesion.

Auramine O stain. X 250.



bronchial lymph node of an animal (bovine) that reacted to tuberculin injected intradermally. Mycobacterium bovis was isolated from this lesion.

Figure 8 illustrates myriads of fluorescing acid-fast bacilli stained with auramine O. These acid-fast bacilli were in the necrotic debris of the same lesion of the same animal described previously.

Figure 9 depicts some fluorescing acid-fast bacilli stained with auramine O. These bacilli were in epithelioid cells and giant cell formations of a lesion of tuberculosis. This lesion was found in the lung of a non-reactor bovine animal on the kill floor. Frequently, lesions suspected of being tuberculous are observed on the slaughterhouse floor by meat inspectors. These animals have not been tuberculin-tested. Mycobacterium bovis was isolated and identified.

A few fluorescing acid-fast bacilli stained with auramine O are evident in Figure 10. The bacilli were observed within epithelioid cells in a lesion of tuberculosis; this lesion was present in the mediastinal lymph node of a bovine reactor animal.

Mycobacterium avium was found in 52 cases.

Figure 11 is a fluorescent photomicrograph of numerous fluorescing acid-fast bacilli in the necrotic debris of a tuberculous lesion. This lesion was found in the mesenteric

Figure 8. Fluorescent photomicrograph of acid-fast bacilli in the necrotic debris of a lesion of tuberculosis in lymph node tissue from a bovine reactor animal. Mycobacterium bovis was isolated from this lesion.

Auramine O stain. X 250.

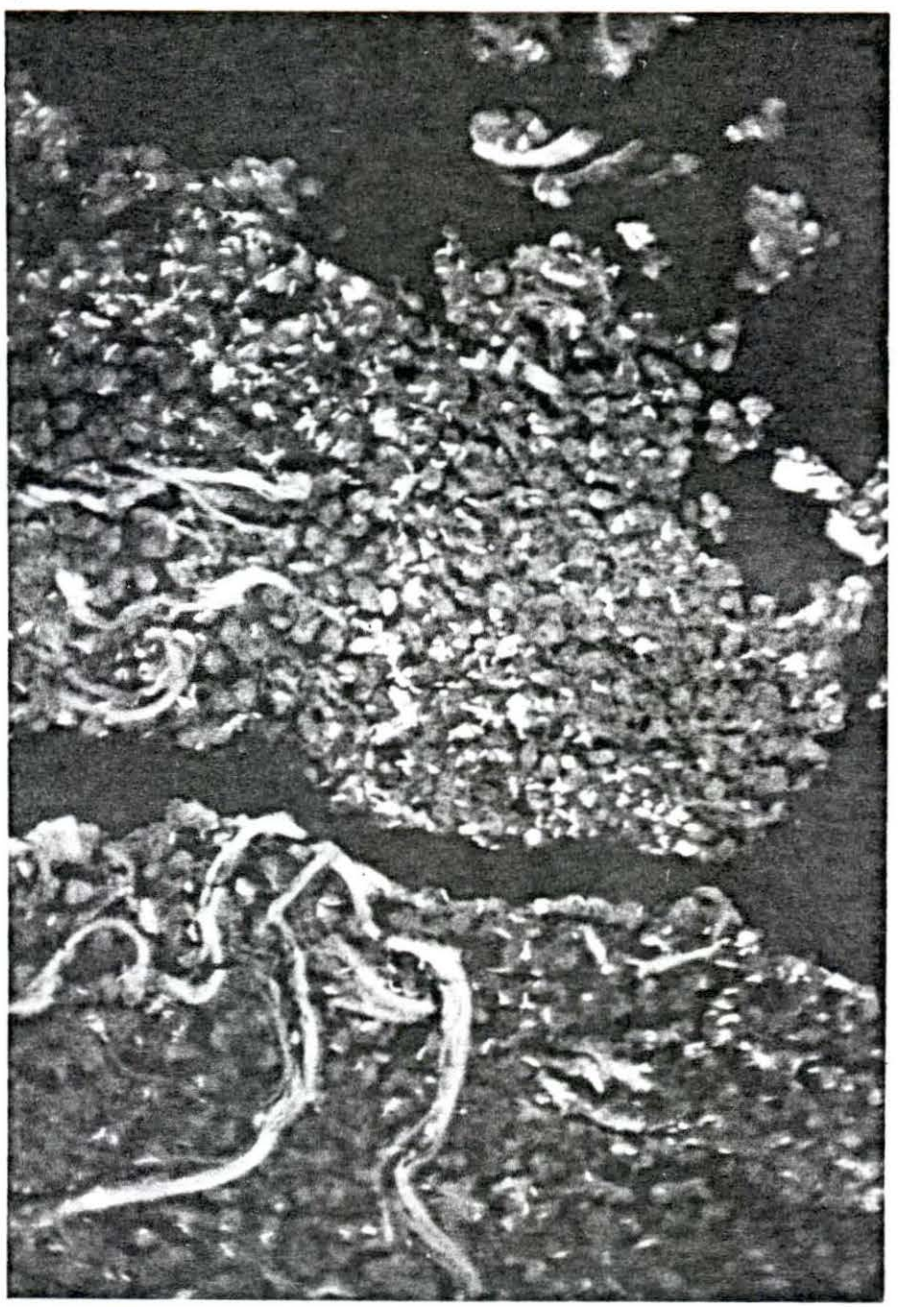


Figure 9. Fluorescent photomicrograph of acid-fast bacilli in epithelioid cells and giant cell formations of a lesion of tuberculosis in lung tissue from a non-reactor bovine animal found on the slaughterhouse kill-floor. Mycobacterium bovis was isolated from this lesion.

Auramine O stain. X 400.

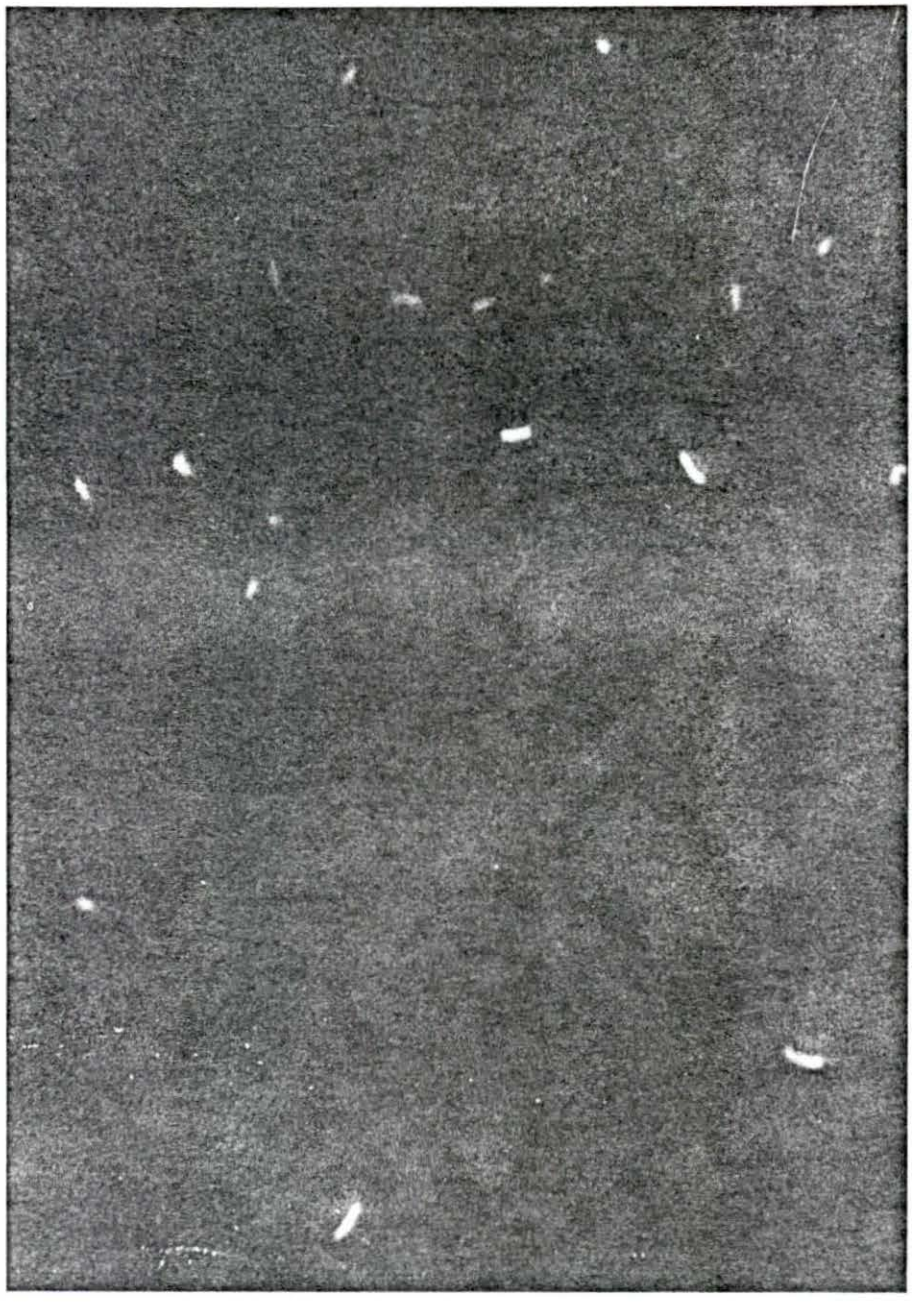


Figure 10. Fluorescent photomicrograph of acid-fast bacilli in epithelioid cells of a lesion of tuberculosis in lymph node tissue from a bovine reactor animal. Mycobacterium bovis was isolated from this lesion.

Auramine O stain. X 400.

Figure 11. Fluorescent photomicrograph of acid-fast bacilli in the necrotic debris of a lesion of tuberculosis in lymph node tissue from a non-reactor bovine animal found on the slaughterhouse kill-floor. Mycobacterium avium was isolated from this lesion.

Auramine O stain. X 250.

lymph node of a non-reactor bovine animal on the slaughter-house kill-floor. Mycobacterium avium was isolated from this lesion.

Mycobacteriologic techniques resulted in the isolation of Mycobacterium tuberculosis in one case, Runyon group III mycobacteria in seven cases, and Runyon group IV mycobacteria in 17 cases.

Group II

The 327 non-lesion cases in this group were negative by fluorescent acid-fast staining with auramine O. Notwithstanding the inability to demonstrate acid-fast bacilli by either conventional (new-fuchsin) or fluorescent (auramine O) staining procedures, mycobacteriologic results included 22 isolations of mycobacteria. Mycobacterium bovis was isolated in ten cases, Mycobacterium avium in six cases, Runyon group II mycobacteria in one case, Runyon group III mycobacteria in one case, and Runyon group IV mycobacteria in four cases.

Group III

All 296 cases in this group contained microscopic lesions either of granulomatous or of non-granulomatous disease conditions upon routine histopathologic examination. Many of

the suggestive tuberculous cases were included in the granulomatous disease conditions.

The overall results of mycobacteriology for this group disclosed several isolations of mycobacteria. These consisted of nine isolations of Mycobacterium bovis, 11 of Mycobacterium avium, one of Runyon group III, and one of Runyon group IV.

Although acid-fast bacilli were not disclosed by conventional (new-fuchsin) staining procedures in any of the 296 cases, 23 cases in this group of 296 were positive by fluorescent acid-fast staining with auramine O.

The difference of 23 cases that were positive by fluorescent acid-fast staining using auramine O, but negative by conventional acid-fast staining using new-fuchsin, were diagnosed histopathologically as follows: 19 cases were suggestive of tuberculosis, three cases were lesions suggestive of migrating parasites, and one case was suggestive of subacute bronchopneumonia.

The 19 cases suggestive of tuberculosis were evaluated further by mycobacteriology. The mycobacteriologic results were as follows: Mycobacterium bovis was isolated from seven cases, and Mycobacterium avium was isolated from five cases. Three cases were mycobacteriologically negative and four cases were not submitted for mycobacteriology because of insufficient tissues.

Fluorescing acid-fast bacilli stained with auramine O are shown in Figure 12. This fluorescent photomicrograph depicts the Mycobacterium bovis bacilli in situ in the necrotic debris of a lesion present in the mediastinal lymph node of a bovine reactor animal.

Figure 13 shows innumerable fluorescing acid-fast bacilli stained with auramine O. These acid-fast bacilli were within the necrotic debris of a lesion in the unknown lymph node of a bovine reactor animal. Figure 14 illustrates fluorescing Mycobacterium avium at a higher magnification in another area in the same lesion as Figure 13.

The three cases that contained lesions suggestive of migrating parasites were submitted to the mycobacteriology section. The mycobacteriologic results were Mycobacterium avium isolants in two cases and Runyon group III isolant in one case.

Several acid-fast coccobacilli were evident in the fluorescent photomicrograph, Figure 15. The fluorescing acid-fast bacilli were observed in the necrotic debris of a suggestive lesion of migrating parasite larvae. The lesion was found in the bronchial lymph node of a bovine reactor animal. Mycobacterium avium was found.

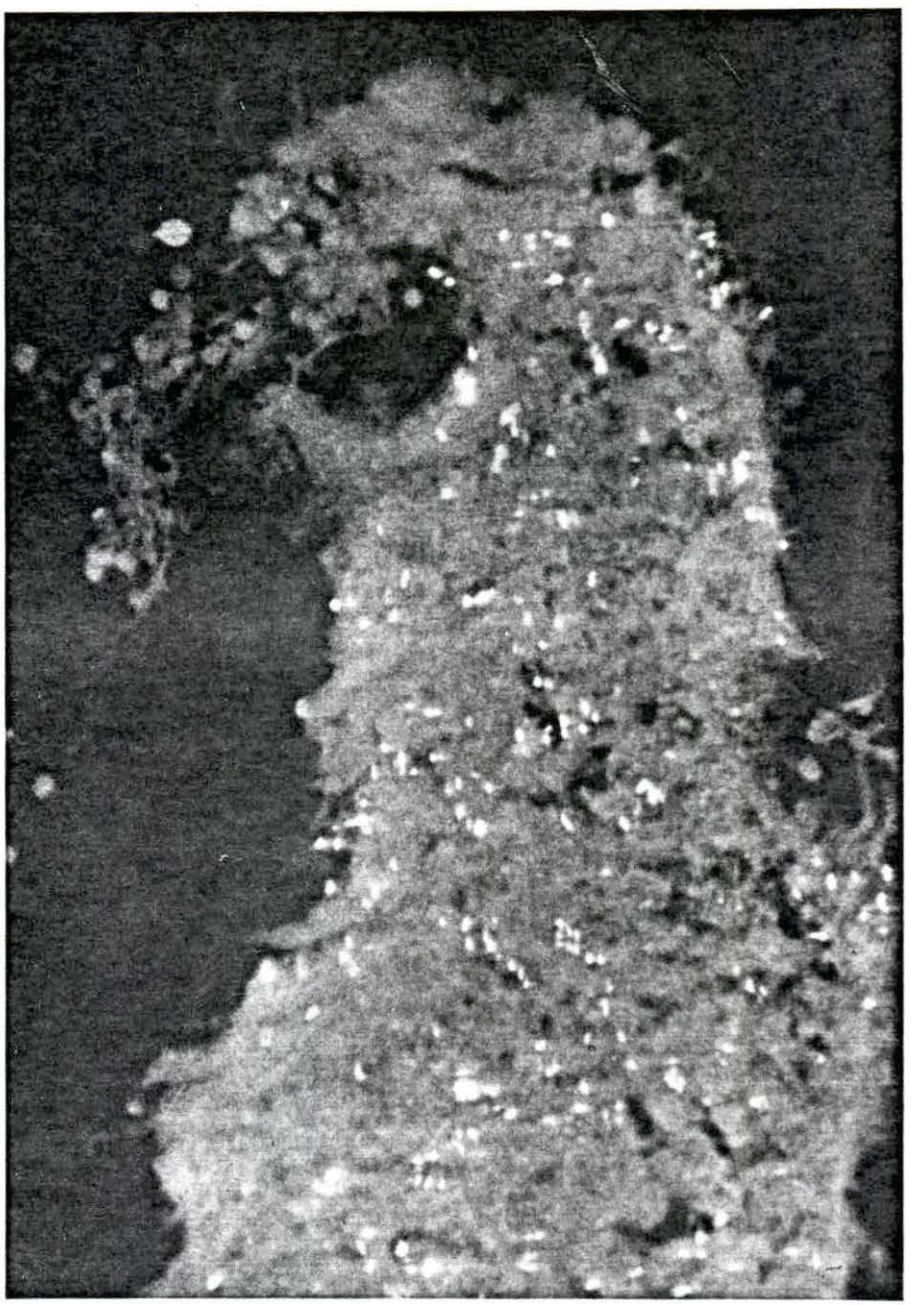
The one case suggestive of subacute bronchopneumonia was submitted to mycobacteriology with the resultant isolation of Mycobacterium avium.

Figure 12. Fluorescent photomicrograph of acid-fast bacilli in the necrotic debris of a lesion of suggestive tuberculosis in lymph node tissue from a bovine reactor animal. Mycobacterium bovis was isolated from this lesion.

Auramine O stain. X 250.

Figure 13. Fluorescent photomicrography of acid-fast bacilli in the necrotic debris of a lesion of suggestive tuberculosis in lymph node tissue from a bovine reactor animal. Mycobacterium avium was isolated from this lesion.

Auramine O stain. X 400.



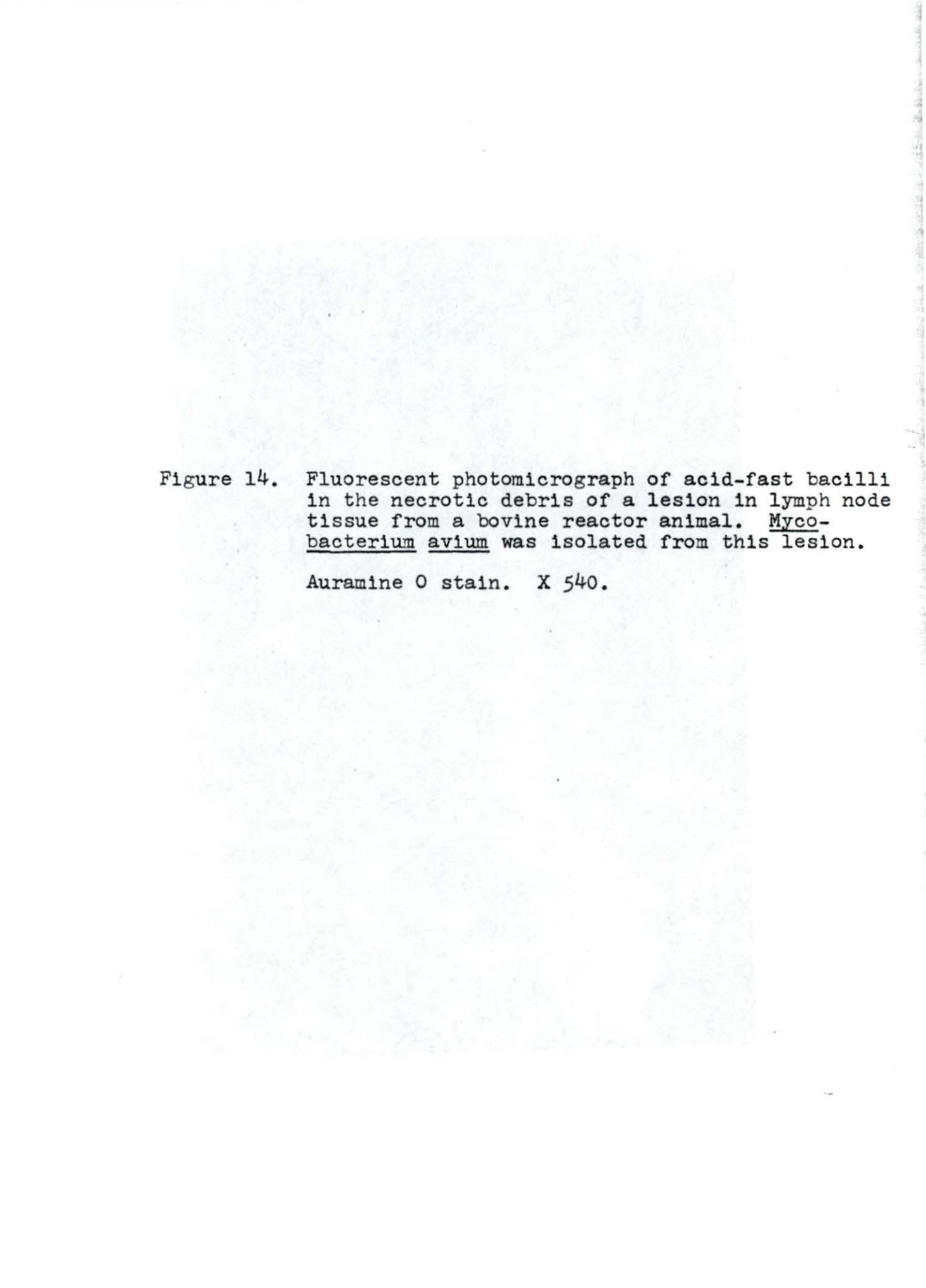
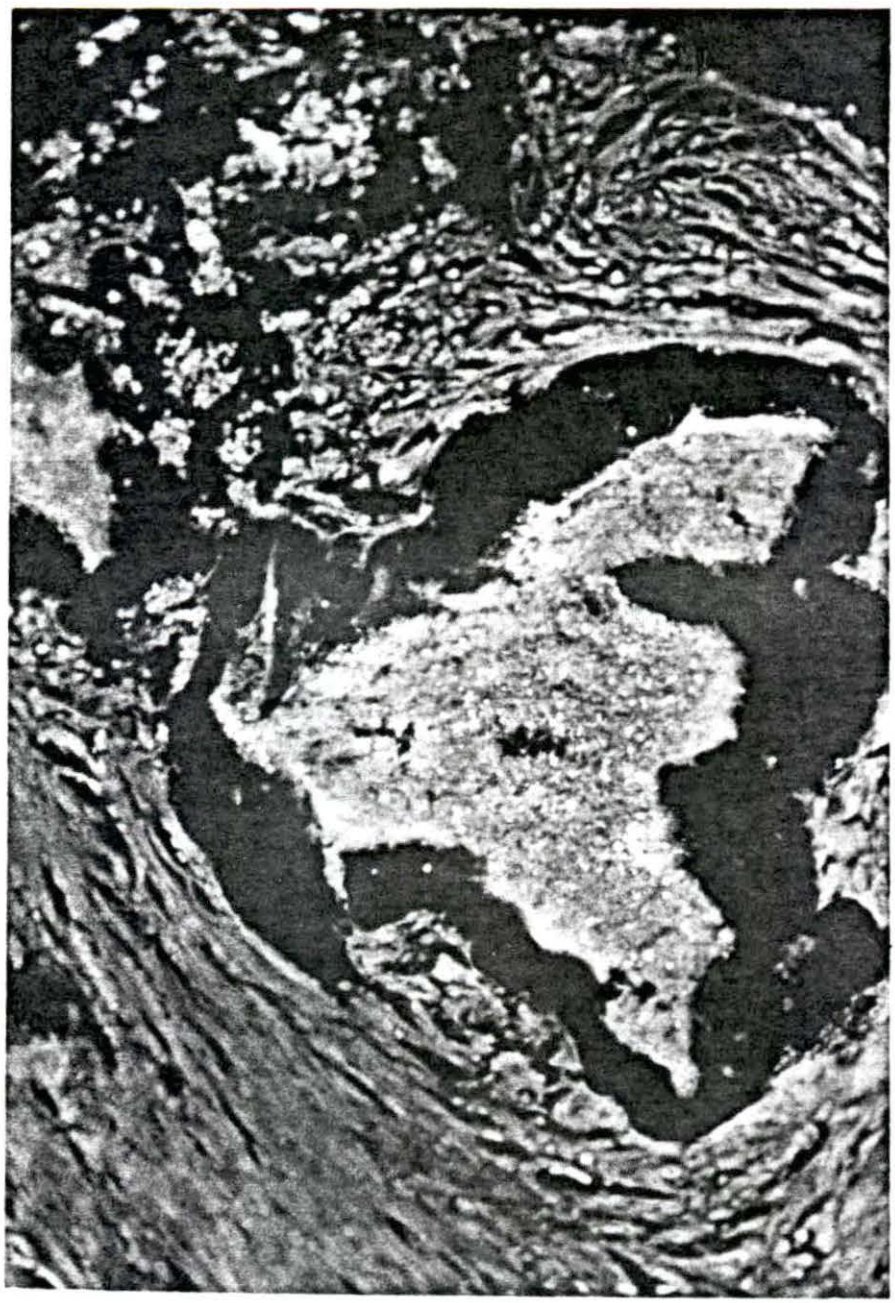


Figure 14. Fluorescent photomicrograph of acid-fast bacilli in the necrotic debris of a lesion in lymph node tissue from a bovine reactor animal. *Mycobacterium avium* was isolated from this lesion.

Auramine O stain. X 540.

Figure 15. Fluorescent photomicrograph of acid-fast bacilli in the necrotic debris of a suggestive lesion of migrating parasitic larvae in lymph node tissue from a bovine reactor animal. Mycobacterium avium was isolated from this lesion.

Auramine O stain. X 125.



Group IV

Fluorescing acid-fast bacilli stained with auramine O were readily demonstrated in several lesions of tuberculosis from two experimentally-produced bovine infections with Mycobacterium bovis. Mycobacterium bovis was again re-isolated from these lesions.

No acid-fast bacilli were evident within representative tissues with fluorescent staining using auramine O in the negative control animal (bovine). All representative tissues from this bovine animal were mycobacteriologically negative.

DISCUSSION

The fluorescent staining procedure employed in this study proved to be a useful, convenient aid for detecting mycobacteria in tissue sections. Using this technique the tissue background faded out to a large extent and bothersome morphological tissue details were minimized microscopically so that the acid-fast bacilli appeared more obvious. This proved to be advantageous in several cases.

In typical cases of Mycobacterium bovis and Mycobacterium avium infections in the bovine, acid-fast bacilli usually can be detected readily during histopathologic examination, but occasionally the acid-fast bacilli are extremely hard to detect. When infections with Runyon group III[1] or other atypical mycobacteria occur in the bovine, the lesions may not be typical of tuberculosis and the acid-fast bacilli may be difficult to find. Furthermore, infections with the atypical mycobacteria may not cause any development of tuberculous lesions in some bovine tissues, such as mesenteric lymph nodes, yet mycobacteria are isolated very readily from apparently normal tissues (49).

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[1]This is a classification of anonymous mycobacteria as proposed by Runyon (48). It is restricted to pulmonary disease in humans in the literature. However, it is being used at the National Animal Disease Laboratory in routine mycobacteriology.

Some of the mycobacteria are capable of invading the tissues and producing extensive lesions in the primary host, such as Mycobacterium bovis in bovine tissues. Some mycobacteria, such as Runyon groups I, II, III, and IV and other saprophytic mycobacteria, such as Mycobacterium smegmatis and Mycobacterium fortuitum, have a limited ability to invade intact healthy tissue but they occur as secondary invaders in lesions initially produced by other agents, such as parasites, other bacterial pathogens, and fungi. Traumatic injury is yet another possibility whereby an initial lesions is infected by mycobacteria. The same situation seems to prevail when one species of mycobacteria occurs in a host other than its primary host, i.e. Mycobacterium avium in the bovine. These secondary invading mycobacteria can produce chronic granulomatous lesions from an initial suppurative or traumatic lesion. In the bovine these secondary mycobacterial infections initiate an immunologic response indistinguishable from the response caused by Mycobacterium bovis on intradermal tests using conventional tuberculin. Histopathologic examination of lesions from such cases does not provide an explanation for the positive intradermal test if the mycobacteria were not found. In some cases the primary cause of the lesion can be readily detected microscopically, but a small number of secondarily-invading or "opportunist" mycobacteria may go undetected.

The greater sensitivity of the fluorescent staining procedure enabled demonstration of acid-fast bacilli in some of these cases that otherwise would have gone undetected and in some of the questionable cases.

In discussing the results of Group I, all 1,111 cases of bovine tuberculosis were positive for acid-fast bacilli upon fluorescent staining with auramine O. These results were attributed in part to the adaptation of some specific features of a number of other non-related fluorescent staining techniques in current use. The specific features that were considered the salient points of many procedures are outlined as follows: the use of a strong acid solution for more complete tissue decolorization of excess, tissue-retained auramine O; the use of ferric chloride solution to quench extraneous, non-specific fluorescence; and the use of a recently developed non-fluorescent mounting medium, Styron 666 in dibutyl phthalate, to eliminate fluorescence attributed to some mounting media.

Some of the techniques had to be modified somewhat so that they could be incorporated for application in this study. These modifications were centered around the adaptation of fluorescent acid-fast staining to bovine lymph node tissues and the strict control of the water content in tissue sections. Both modifications were achieved by thorough deparaffinization and dehydration procedures.

The mycobacteriologic isolations from approximately one-half of the cases under study in Group I were within those average results obtained in a review of the literature (8, 37, 38, 39, 40, 49).

The 327 non-lesion cases in Group II were negative by fluorescent acid-fast staining with auramine O; however, mycobacteria were isolated in 22 cases. This is in accord with the work of Mallmann (37, 38) and Smith (49) in which they frequently isolated Mycobacterium bovis from histopathologically normal bovine lymph node tissues. Workers at the National Animal Disease Laboratory have found that mycobacteria are isolated from apparently normal tissues (8, 40).

Out of 296 cases in Group III classified either as granulomatous or as non-granulomatous disease conditions, fluorescent acid-fast staining revealed 23 cases that were positive with acid-fast bacilli. Mycobacteriologic results included seven isolations of Mycobacterium bovis, eight of Mycobacterium avium, one of Runyon group III, and three negative. Four cases were not submitted for mycobacteriology because of insufficient tissues.

The other 273 cases in this group were cultured for mycobacteria with the resultant isolations of Mycobacterium bovis in two cases and Mycobacterium avium in three cases.

While many cases, positive by fluorescence microscopy but negative with the acid-fast stain, have been confirmed

as mycobacteriologically positive, a fair number of cases are on record in which any such confirmation was lacking altogether. Some people have, therefore, concluded that the fluorescent method is not sufficiently specific and therefore have advised against its use. Such conclusions are premature because the following possible causes may explain an apparent or alleged non-specificity in a given case. One cause may be the heterogenicity of samples; there may have been no organisms present in the adjacent material subjected to mycobacteriologic work. Furthermore, there may have been a loss of viability of microscopically demonstrable tubercle bacilli. This may occur in pre-treated specimens or where the bacilli are dead in the tissue due to the buildup of toxic metabolic products or to anaerobiosis.

The problem of fluorescing artefacts must be carefully considered. This problem has been practically eliminated by the quenching of extraneous fluorescence with ten percent ferric chloride and other suitable quenching agents. Moreover, artefacts need not be a problem if the following criteria are carefully applied. Typical size of acid-fast bacilli is a prime consideration; most acid-fast organisms measure two to five micra in length and 0.2 to 1.5 micra in width. Generally speaking, most artefacts are larger. Typical form is another consideration; acid-fast bacilli are

generally present as narrow rods or short, regular coccobacilli which are sometimes curved whereas most artefacts are plump, irregular masses. Typical structure is yet another consideration; acid-fast bacilli are either homogeneous or granular in appearance. This is in contradistinction to artefacts which are either heterogeneous or amorphous in character. Sharp delineation of acid-fast bacilli is an important differential feature; artefacts have fuzzy, ill-defined borders. Most acid-fast bacilli show strong and brilliant fluorescence and many artefacts exhibit weak and variable fluorescence.

In discussing Group IV, fluorescing acid-fast bacilli stained with auramine O were easily demonstrated in several lesions of tuberculosis from the two experimentally-produced bovine infections with Mycobacterium bovis. This organism was again re-isolated from these lesions. Thus, Koch's postulates were completely fulfilled.

A negative control animal was utilized in this study. No acid-fast bacilli were evident within representative tissue fluorescent staining with auramine O. All representative tissues from this animal were negative.

Finally, some overall viewpoints pertaining to this study merit discussion.

Experience has shown this fluorescent staining procedure incorporating auramine O to be efficient, to be

economical, and to be highly advantageous for the following reasons in comparison to the time-honored acid-fast staining procedures. The staining procedure is simpler as one can readily determine for himself when one compares the respective acid-fast staining techniques. The staining time is reduced by approximately one-fourth. The fluorescing organisms offer much greater contrast, appearing as brilliant golden-yellow or yellow, self-luminous bacilli or coccobacilli against a darkened background. This greatly facilitates the demonstration of a single bacillus. The organisms may be detected at low magnification by their dispersion halo, even when the organisms are slightly out of focus.

Oil immersion objective lenses need not be employed for fluorescence microscopy. Instead, the fluorescent bacilli are searched for under medium dry, or even low dry, magnification and then conclusively identified with the medium dry or high dry lenses. Therefore, it is apparent that a much larger area may be covered, not only in width but also in depth.

Average scanning time is considerably shortened, often by as much as one-half or more. It appears that the more difficult the acid-fast bacilli are to be found, which apparently may be a function of the relative numbers of acid-fast bacilli present, the more the scanning time is shortened by using the fluorescent method; this is in

comparison to the conventional acid-fast staining techniques. Bogen (5) stated that while all parts of an auramine O stained slide may be examined in less than 20 minutes, it may take more than eight hours to accomplish this in an acid-fast stained slide under oil immersion.

An advantage that is very obvious but not readily apparent is that color-blind personnel may use this method without difficulties.

More positive cases of tuberculosis are found with the fluorescent method because its sensitivity exceeds that of any other microscopic technique with which it has been compared (28). This increased sensitivity is explained by a number of observations that are discussed in the following paragraphs.

More bacilli are stained by the fluorescent dyes, including auramine O, than by carbol-fuchsin as shown in ruled fields of identical slides successively stained with auramine O after destaining the conventional acid-fast techniques (43, 45).

Mycolic acid, the principal acid-fast component of tubercle bacilli, combines more readily with auramine O than with carbol-fuchsin and, in the former combination, resists destaining for a much longer time. Thus, it is likely that the demonstration of acid-fast organisms by fluorescence microscopy includes a larger number of bacilli

with low or lower mycolic acid content than normal, such as saprophytic mycobacteria, young tubercle bacilli, and dead tubercle bacilli (43).

Finally, the larger microscopical area under observation and the comparative ease with which the fluorescent bacilli are detected contribute to its increased sensitivity.

Staining experiments by Lempert (35), Finke (16), Richards (43), and others indicate that the fluorochrome, auramine O, is specific for the acid-fast group of bacilli.

It must be borne in mind that discrepancies between histopathologic and mycobacteriologic data are not only found with fluorescence microscopy but also with the conventional acid-fast procedures. In fact, low positive results with the latter procedures are frequently combined with negative cultures, and yet the Ziehl-Neelsen technique is considered reliable and time-honored.

Another difficulty that the author encountered in this study was the problem of the fluorescence fading out of the auramine-stained acid-fast bacilli in the tissue sections. Subjectively speaking, it was apparent that the auramine O fluorescence was noticeably fading six days after being stained and coverslipped. A cursory review of slides that were at least 60 days old revealed almost a complete loss of adequate fluorescence. This has been mentioned in the human medical records as a problem.

Other practical difficulties are evident in the fluorescent staining method also. Fluorescence microscopy requires a certain degree of skill; this skill is gained only after continual utilization of this method. For this reason complete confidence is not easily and quickly gained. Any changeover from conventional staining of acid-fast bacilli to fluorescent staining should be made over a considerable length of time. Allowing for gradual adjustment, efficiency will improve considerably.

Focusing is another difficulty that has been encountered in this study. This is almost totally attributable to the dark background of the darkfield. However, it has been found that by carefully focusing at the edge of the lesion under consideration that this difficulty is lessened. Also, it has been noted that by working in a darkened room, or a room with subdued lighting, that the problem of focusing is minimized. It is important to allow the light source to perform to full capacity before beginning the examination.

CONCLUSIONS

This study was designed to test the thesis that the fluorescent staining of acid-fast bacilli in bovine tissues with auramine O was feasible. Therefore, an experimental design to test this thesis was organized. To achieve this end, an investigation was conducted on 1,737 cases divided into four groups. Group I consisted of 1,111 cases classified histopathologically as compatible with tuberculosis. Group II was composed of 327 cases that were histopathologically negative or non-lesion cases. Group III consisted of 296 cases containing lesions histopathologically indicative of both granulomatous and non-granulomatous disease conditions including some suggestive tuberculosis. Group IV consisted of two experimentally-produced cases of Mycobacterium bovis infection and one negative control case.

Group I

All of the 1,111 tuberculous cases in this group were positive upon fluorescent acid-fast staining with auramine O. Mycobacteriologic techniques resulted in the isolation and identification of Mycobacterium bovis in 452 cases, Mycobacterium avium in 52 cases, Mycobacterium tuberculosis in one case, Runyon group III mycobacteria in seven cases, and Runyon group IV mycobacteria in 17 cases. The remaining 592 cases in this group either were not cultured for mycobacteria

because of insufficient tissue or were mycobacteriologically negative.

Group II

The 327 non-lesion cases in this group were negative by fluorescent acid-fast staining with auramine O. Mycobacteriologic results included 22 isolations of mycobacteria. Mycobacterium bovis was isolated in ten cases, Mycobacterium avium in six cases, Runyon group II mycobacteria in one case, Runyon group III mycobacteria in one case, and Runyon group IV mycobacteria in four cases.

Group III

Of 296 cases, fluorescent acid-fast staining with auramine O disclosed 23 cases that were positive with acid-fast bacilli which were originally classified either as granulomatous or as non-granulomatous disease conditions. These cases were classified as such because acid-fast bacilli were not demonstrable by conventional means.

Among the 23 cases were 19 suggestive tuberculosis cases, three suggestive parasitic cases, and one subacute bronchopneumonia case.

In the 19 cases suggestive of tuberculosis, Mycobacterium bovis was isolated from seven cases, and Mycobacterium avium was isolated from five cases. Three cases were mycobacteriologically negative and four cases were not submitted for

mycobacteriology because of insufficient tissues. The mycobacteriologic processing resulted in Mycobacterium avium isolants in two cases and Runyon group III isolant in one case of suggestive parasitic lesions.

Mycobacteriology in the subacute bronchopneumonia case provided the resultant isolation of Mycobacterium avium.

The overall isolations from this particular group were as follows: nine were Mycobacterium bovis, 11 were Mycobacterium avium, one was Runyon group III, and one was Runyon group IV.

Group IV

Fluorescing acid-fast bacilli stained with auramine O were readily demonstrated in several lesions of tuberculosis from two experimentally-produced bovine infections with Mycobacterium bovis. Mycobacterium bovis was again reisolated from these lesions.

No acid-fast bacilli were evident within representative tissues with fluorescent staining using auramine O in the negative control animal (bovine). All representative tissues from this bovine animal were mycobacteriologically negative.

The observations in this investigation were in accord with those reported in human medicine in regard to the application of fluorescent staining for the detection of acid-fast bacilli in tissue sections. Fluorescent staining

with auramine O was an efficient and sensitive procedure to detect acid-fast bacilli in tissue sections of bovine specimens suspected of being tuberculous. Therefore, fluorescent acid-fast staining is to be recommended for application in veterinary medicine in the realm of bovine tuberculosis.

Once the fluorescent staining technique has been mastered, fluorescence microscopy has the potential to serve as a highly useful tool in the detection of acid-fast bacilli in tissue sections. This is by virtue of its technical simplicity and the ease of observation afforded. There was no reasonable increase in the time spent in the overall aspect of tissue preparation, tissue staining, and tissue examination as compared with the conventional staining procedures. Also, information can be gained by this method of acid-fast staining which may be unobtainable by other conventional acid-fast staining procedures.

Difficulties encountered in this investigation were centered around the problems of the fluorescence fading out of the auramine-stained slides after about six days. The problem of focusing upon the tissue was an extremely critical matter that required much time and patience on the part of the observer.

LITERATURE CITED

1. Arloing, F., Policard, A., and Langeron, L. Aspects offerts par livers cultures microbiennes sous l' action des radiations emises par la lampe a arc au mercure apres filtration par des ecrans de wood. Comptes rendus des seances de la Societe de biologie 92:261-263. 1925.

2. Armed Forces Institute of Pathology. Manual of histology and special staining technics. Washington, D. C., Author. 1957.

3. Bachmann, W. and Finke, L. Uber die fluoreszenz-mikroskopische darstellung von tuberkelbazillen im gewebe. Deutsche medizinische Wochenschrift 65: 1474-1475. 1939.

4. Bekker, J. H. and Tasman, A. Uber den zusammenhang zwischen der sauerfestigkeit und dem fluoreszenz-vermoegen des tuberkelbazillus. Archiv fur Hygiene und Bakteriologie 127:7-9. 1941.

5. Bogen, E. Detection of tubercle bacilli by fluorescence microscopy. American Review of Tuberculosis 44:267-271. 1941.

6. Boyd, W. A textbook of pathology. 7th edition. Philadelphia, Pennsylvania, Lea and Febiger. 1961.

7. Briggs, G. O. A. and Jennison, M. H. Detection of Mycobacterium tuberculosis by means of fluorescence microscopy. Tubercle 28:189-192. 1942.

8. Cassidy, D. R., Morehouse, L. G., and McDaniel, H. A. Mycobacterium avium in the bovine. Unpublished manuscript. Ames, Iowa, U. S. Department of Agriculture, Agricultural Research Service, ca. 1966.

9. Clark, R. F. and Hench, M. E. Practical applications of acridine orange stain in the demonstration of fungi. American Journal of Clinical Pathology 37: 237-238. 1962.

10. Clauberg, K. W. Uber die verwendbarkeit des fluoreszenzmikroskopes in der bakteriologischen praxis, zug leich uber die frage des vorkommens virusartiger microorganisem in menschlichen kregsgewebe. Klinische Wochenschrift 18:632-635. 1939.

11. Clegg, J. W. and Foster-Carter, A. F. Detection of tubercle bacilli by fluorescence technique. British Journal of Tuberculosis 40:98-105. 1946.

12. Crossman, G. and Lowenstein, E. Demonstration of tubercle bacilli in tissue by fluorescence microscopy. Journal of Laboratory and Clinical Medicine 28: 1349-1354. 1943.

13. De Gommier, J. Fluorescence et acido=alcoolo= resistance relative du bacille tuberculeux. Annales de l'Institut Pasteur 96:723-733. 1959.

14. Didion, H. Uber den fluoreszenzmikroskopischen nachweis von tuberkelbacterien. Klinische Wochenschrift 18:1315-1318. 1939.

15. Dienst, R. B. The use of the fluoroscopic method for diagnosis of Mycobacterium tuberculosis in sputum. American Journal of Clinical Pathology 6:61-62. 1942.

16. Finke, L. Uber den fluoreszenzmikroskopischen nachweis von tuberkelbazillen im ausstrichen und gewebsschnitten. Archiv fur Hygiene und Bakteriologie 123:381-392. 1940.

17. Freiman, D. G. and Mokotoff, G. F. Demonstration of tubercle bacilli by fluorescence microscopy. American Review of Tuberculosis 48:435-442. 1943.

18. Gastambide-Odiers, M. and Smith, D. W. A rapid means of differentiating atypical acid-fast bacilli and avian tubercle bacilli from other mycobacteria. American Review of Tuberculosis 77:662-668. 1959.

19. Gilkerson, S. W. and Kanner, O. Improved technique for the detection of acid-fast bacilli by fluorescence. Journal of Bacteriology 86:890-891. 1963.

20. Graham, C. F. Fluorescence microscopy of Mycobacterium tuberculosis. American Review of Tuberculosis 48: 421-434. 1943.

21. Gunn, F. D. Tuberculosis. In Anderson, W. A. D., ed. Pathology 4th ed. pp. 243-263. Saint Louis, Missouri, C. V. Mosby Co. 1961.

22. Hagemann, P. K. H. Fluoreszenzmikroskopische untersuchungen uber virus und andere mikroben. Zentralblatt fur Bakteriologie, Parasitenkunde und Infektionskrankheiten 140:184-187. 1937.

23. Hagemann, P. K. H. Fluoreszenz-farbung von tuberkelbakterien im auramin. Munchener medizinische Wochenschrift 85:1066-1068. 1938.

24. Haitinger, M. and Hamperl, H. Die anwendung des fluoreszenzmikroskops zue untersuchung tierischer gewebe. Zeitschrift fur mikroskopischanatomische Forschung 33: 193-221. 1933.

25. Hauduroy, P. and Posternak, J. Les applications bacteriologiques de la microscopie en fluorescence. Recherches sur la microscopie en fluorescence du bacille tuberculeux. Schweizerische Zeitschrift fur Pathologie und Bakteriologie 5:240-263. 1942.

26. Herrmann, W. Der nachweis von tuberkelbazillen mit dem fluoreszenzmikroskop. Deutsche medizinische Wochenschrift 64:1354-1356. 1938.

27. Hughes, G. C. Improved stain for fluorescence microscopy. Tubercle 27:91-92. 1946.

28. Kanner, O., Gilkerson, S. W., and Hogue, C. Detection of acid-fast bacilli by a technique of specific fluorescent staining. U. S. Veterans Administration Research Conference in Pulmonary Diseases Transactions 22:108-109. 1963.

29. Karlson, A. G., Martin, J. K., Jr., and Harrington, R., Jr. Identification of Mycobacterium tuberculosis with one tube of liquid medium. Mayo Clinic Proceedings 39: 410-415. 1964.

30. Keller, C. J. Vereinfachter nachweiss von tuberkelbazillen im fluoreszenzlicht. Munchener medizinische Wochenschrift 85:2024-2028. 1938.

31. Koch, M. L. and Cote, R. A. Comparison of fluorescence microscopy with Ziehl-Neelsen stain for demonstration of acid-fast bacilli in smear preparations and tissue sections. American Review of Respiratory Diseases 91: 283-284. 1965.

32. Kohler, A. Mikrophotographische untersuchungen mit ultravioletten licht. Zeitschrift fur wissenschaftliche mikroskopie und mikroskopische Technik 21: 129-165. 1904.

33. Kuster, H. Untersuchungen mit dem fluoreszenzmikroskop. Deutsche medizinische Wochenschrift 65:92-94. 1939.

34. Lee, H. I. Comparison of procedures for staining tubercle bacilli in fluorescence microscopy. Journal of Laboratory and Clinical Medicine 29:218-221. 1944.

35. Lempert, H. Fluorescence microscopy in the detection of tubercle bacilli. Lancet 247:818-822. 1944.

36. Lind, H. E. and Shaughnessy, H. J. Fluorescent staining technique for detection of acid-fast bacilli. Journal of Laboratory and Clinical Medicine 27: 531-536. 1947.

37. Mallmann, W. L. A progress report of tuberculosis research at Michigan State University. U. S. Livestock Sanitary Board Proceedings 65:174-178. 1961.

38. Michigan State University. Departments of Microbiology and Public Health and Veterinary Pathology. Bovine tuberculosis. Fourth semi-annual progress report of the research contract with the U. S. Department of Agriculture (June 30, 1961). 1961.

39. Michigan State University. Departments of Microbiology and Public Health and Veterinary Pathology. Bovine tuberculosis. Third semi-annual progress report of the research contract with the U. S. Department of Agriculture (December 31, 1960). 1960.

40. Morehouse, L. G., Singer, R. H., McDaniel, H. A., Howell, D. G., Sherman, K. C., and Cassidy, D. R. Examination of bovine specimens suspected of being tuberculous. U. S. Livestock Sanitary Board Proceedings 67:430-437. 1963.

41. Oscarsson, P. N. Zur frage des wertes der fluoreszenzmikroskopie fur den nachweis von tuberkelbazillen im sputum. Acta medica Scandinavica 108:240-250. 1941.

42. Petrini, M. Sulla diagnosi batteriologica di tuberculosi umana mediante la microscopia a fluorescneza. Giornale di Batteriologia e Immunologia 34:137-148. 1946.

43. Richards, O. W. The staining of acid-fast tubercle bacteria. Science 93:190. 1941.

44. Richards, O. W., Kline, E. K., and Leach, R. E. Demonstration of tubercle bacilli by fluorescence microscopy. American Review of Tuberculosis 44: 255-266. 1941.

45. Richards, O. W. and Miller, D. K. An efficient method for the identification of Mycobacterium tuberculosis with a simple fluorescence microscope. American Journal of Clinical Pathology 5:1-8. 1941.

46. Rihl, H. Erfahrungen uber den tuberkelbazillen nachweis mit dem fluoresmikroskop. Wiener klinische Wochenschrift 53:322-323. 1940.

47. Robbins, S. L. Textbook of pathology with clinical application. 2nd edition. Philadelphia, Pennsylvania, W. B. Saunders Co. 1962.

48. Runyon, E. H. Anonymous mycobacteria in pulmonary disease. Medical Clinics of North America 43: 273-290. 1959.

49. Smith, H. W. The isolation of mycobacteria from mesenteric lymph-nodes of domestic animals. Journal of Pathology and Bacteriology 68:367-362. 1954.

50. Tanner, F. H. Fluorescence method of demonstrating Mycobacterium tuberculosis in tissues. American Journal of Medical Technology 14:83-86. 1948.

51. Tanner, F. H. Fluorescence microscopy for demonstrating Mycobacterium tuberculosis in tissues. Mayo Clinic Staff Meetings Proceedings 16:839-842. 1941.

52. Thompson, L. Comparison of carbol fuchsin with the fluorescent dye auramine for the demonstration of acid-fast bacteria. Mayo Clinic Staff Meetings Proceedings 16:673-675. 1941.

53. Thompson, L. Reliability of auramine stain for Mycobacterium tuberculosis. Mayo Clinic Staff Meetings Proceedings 18:163-164. 1943.

54. Van Dyke, A. E. A comparative evaluation of Ziehl-Neelsen and fluorescent methods in demonstrating acid-fast bacilli. American Journal of Clinical Pathology Technical Supplement 7:6-8. 1943.

55. Von Haebler, T. and Murray, J. F. Fluorescence microscopy as a routine method for detection of Mycobacterium tuberculosis and Mycobacterium leprae. South African Medican Journal 28:45-48. 1954.

56. Willigan, D. A., Garric, V. A., and Trasko, B. K. New fuchsin-hematoxylin-eosin; a stain for acid-fast bacilli and surrounding tissue. Stain Technology 36: 319-321. 1961.

ACKNOWLEDGEMENTS

The author is indebted to Dr. F. K. Ramsey, Dr. C. C. Bowen, Dr. D. R. Cassidy, Dr. M. L. Kaeberle, Dr. L. H. Tiffany, and members of the staff of Diagnostic Services at the National Animal Disease Laboratory for their generous assistance during the course of this investigation and the preparation of this thesis.

The writer would especially like to acknowledge the constant encouragement and technical assistance of my dear wife, Martha P. Howell.

The helpful editorial comments of Dr. F. K. Ramsey and Dr. D. R. Cassidy are gratefully acknowledged.

The technical assistance of Mrs. E. J. Henry, Miss K. O. Teig, and Mr. R. M. Glazier is greatly appreciated. An especial acknowledgement is in order to Dr. W. D. Yoder and his staff for their technical assistance in the mycobacteriologic typing procedures.