

METHODS FOR THE ISOLATION OF MYCOBACTERIA FROM THE SOIL

by

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## INTRODUCTION

Since long ago, it has been believed that many cases of non-specific positive tuberculin reaction among animals are due to the presence of certain members of the genus mycobacterium other than Mycobacterium tuberculosis. Some of these organisms, the so-called unclassified mycobacteria, whether pathogenic or non-pathogenic are able to sensitize the body of the host leading to a false positive tuberculin test. Many workers isolated a variety of these anonymous mycobacteria from different sources, including soil, grass, leaves, water, and animal excreta. It was found that diseases caused by some members of the unclassified mycobacteria are not communicable, and hence was the belief that there must be a certain reservoir where these organisms can exist in nature other than man. In the present study soil is considered the most probable natural reservoir of the unclassified acid-fast bacilli. Several methods of isolating these organisms from soil in pure culture have been used by research workers in the past. These different methods were compared as to their ability to detect and isolate mycobacteria from the soil in pure culture. In addition several modifications of isolation procedures were designed and tested. These procedures had as their fundamental basis the partition of the bacteria between a hydrocarbon and an aqueous phase. This separation was postulated because of the lipid nature of the cell surface of acid-fast bacteria.

## REVIEW OF THE LITERATURE

The work on saprophytic acid-fast bacteria dates back to 1885 when Alvarez and Tavel (1883) (cited by Frey and Hagan, 1931), isolated an organism of this type from smegma, and called it Bacillus smegmatis. This organism, now called Mycobacterium smegmatis, was described at about the same time by Matterstock (1885). By the intraperitoneal injection of guinea pigs, Lydia Rabinowitsch (1897) isolated the so-called "butter bacillus" (Mycobacterium berolinense). Moeller (1898) was able to isolate two acid-fast organisms; the first was isolated from timothy hay, and was described as the "timothy hay bacillus or grass bacillus No. 1" (Mycobacterium phlei), and the second as the "mist bacillus" (Mycobacterium stercusis), since he isolated it from the dejecta of cows, horses, goats, swine, and mules. Moeller (1899) described a third organism which he had isolated from plant dust as "grass bacillus No. 2" (Mycobacterium graminis). Kersten (1909) (cited by Frey and Hagan, 1931), by using the antiformin method of Uhlenhuth and Kersten, isolated an acid-fast organism from the soil (Mycobacterium alluvialum). Söhngen (1913) succeeded in isolating acid-fast bacteria from the soil, excrement of animals, and stagnant water. Buttner (1926), using the method of Söhngen, found acid-fast bacteria existing in various natural substances, namely, earth, hay, leaves, stagnant water, manure, the contents of cow's stomach, and peat. Of seventeen materials examined only four yielded negative results.

Frey (1930) described a method for detecting acid-fast bacteria in soil. He used carbon-free enriched medium in which paraffin was



added. Incubation was carried out at a high temperature (47.5C) to eliminate mold contamination. Gentian violet in a dilution of 1 to 10,000 was used to inhibit contaminants and aid in the recovery of acid-fast bacteria in pure culture.

Frey and Hagan (1931) using a technique devised by Söhnngen, were able to demonstrate the presence of acid-fast bacteria in a hundred samples of soils collected from various parts of the United States. Of these, twenty-five organisms isolated in pure culture were placed in three rough groups on the basis of cultural features. By means of cutaneous tests on sensitized or immunized guinea pigs, it has been shown that the organisms of any one of these groups exhibit a closer relationship to each other than to organisms of the other groups.

The method used by Frey and Hagan proved reasonable for the isolation of thermophilic mycobacteria. Later study, however, of a larger collection of acid-fast saprophytes showed that many were incapable of growth at 47.5C. This finding called the attention of Gordon and Hagan (1937) to the probability that such microorganisms existed in soil and were being missed by that technique because of their inability to develop at the high temperature used for elimination of molds. Instead, they used a nitrogen-free medium (Ashby's medium) in which glycerol was substituted for mannitol for their first enrichment. Inoculated media were incubated at 25C for 7-10 days. Concentration of acid-fast organisms was achieved by inoculating growth from Ashby's medium into Söhnngen's medium which was supplied with paraffin as a carbon source, and incubated at 37C for 7-10 days. They were able to recover pure cultures by inoculating growth from Söhnngen's media

into solid Dorset's synthetic medium. Twenty-seven out of sixty pure cultures isolated proved incapable of growing at 47.5C, and therefore, could not have been isolated with the technique of Frey and Hagan.

Segard and Thompson (1952) isolated two saprophytic acid-fast bacilli from chlor-phenol red, and a 4% sodium hydroxide solution, respectively, during processing samples in the examination for tubercle bacilli. They described the organism isolated from chlor-phenol red as a rapid growing pigmented acid-fast, short, gram-negative, pleomorphic bacillus which showed acid-fastness on smears from the dye, but not so on culture. The other organism isolated from the sodium hydroxide solution was described as a slow growing, pigmented, acid-fast, gram-positive rod, demonstrable in culture and was not identical with Mycobacterium tuberculosis.

Chapman and Bernard (1963) reported that in Dallas, Texas dairy cattle demonstrated weak reactions to large amounts of tuberculin although they did not show gross lesions. The authors reported that the same condition existed in other areas. They were able to isolate unclassified mycobacteria from raw milk. Of 150 samples, mycobacterial colonies were found in 50. Colonies appeared in 10-28 days. At least six were established as photochromogenic. Others were scotochromogenic or non-chromogenic.

Jefferies and associates (1963) in Florida isolated unclassified mycobacteria from the soil and dust samples. Eighty-two and one-half per cent of the soil samples, and 7.6 per cent of dust samples were positive for unclassified acid-fast bacilli, many of the specimens

showing two or three groups. The predominant type was Runyon's Group ii, Group iii being obtained in almost equal numbers. Group iv was found with moderate frequency.

Recently, Kubica, Beam, and Palmer (1963) described an improved method for the isolation of unclassified acid-fast bacilli from soil. They were able to develop a new decontaminating solution for processing of soil samples by which nearly 50 per cent of the used samples yielded acid-fast bacilli on culture. This decontaminant is composed of 6 volumes of 1.17% sodium hydroxide solution, and 1 volume of a commercial sodium hypochlorite solution that contains 5-6 per cent available chlorine.

Acid-fast microorganisms other than typical tubercle bacilli have been recovered from animal tissue. Hastings, Beach and Thompson (1930) isolated saprophytic acid-fast bacilli from the tissues of cattle which had reacted to the tuberculin test but which presented no gross evidence of tuberculosis at necropsy. With their cultures they were able to produce a sensitivity to tuberculin in tuberculosis-free cattle. Daines (1938) isolated acid-fast bacteria unlike tubercle bacilli from cattle with the so-called skin lesions, and with them was able to produce a tuberculin sensitivity in cattle.

Karlson and Feldman (1940) isolated and described an acid-fast organism from swine tonsils. The microorganism produced no recognizable disease in chickens, mice or calves. Subcutaneous injections of large doses in guinea pigs and rabbits produced a localized region of caseation necrosis with no tendency towards extension of the lesion. The microorganism sensitized guinea pigs to avian tuberculin and to

homologous culture filtrates. No sensitivity to mammalian tuberculin was produced. Cross agglutination reactions indicated that the swine tonsil microorganism had antigenic components in common with the avian tubercle bacillus.

From 100 apparently healthy cattle slaughtered in abattoirs, Smith (1958) isolated unidentified acid-fast bacilli from the retropharyngeal lymph gland in three animals, and from the ileocaecal lymph gland in one animal.

Among 94 sacrificed oxen and cows with positive tuberculin test, Ochi, Yuichi and Heiji Sato (1961) in Japan, were able to isolate 29 strains of non-pathogenic mycobacteria from 16 of these slaughtered animals. More than half the isolated strains were non-photochromogenic.

The current interest in the unclassified mycobacteria is a result of their recognition as potential causes of human disease. The existence of acid-fast bacilli other than Mycobacterium tuberculosis, apparently producing pulmonary disease in humans has been reported sporadically for many years.

Branch (1932-1933) (cited by Steenken, Jr. and Landau, 1936) called attention to a number of acid-fast organisms not of mammalian type isolated from human sources, some of which resembled the avian type. He considered them to be new strains of pathogenic acid-fast organisms. Cummins and Williams (1933-1934) (cited by Steenken, Jr. and Landau, 1936) isolated an acid-fast organism other than Koch bacillus, called the "M" strain, from the sputum of a girl suffering from a chronic pulmonary illness.

Warring, Jr. and Rilance (1943) isolated and studied a saprophytic acid-fast organism, to which the term "yellow bacillus" was applied, from two patients with disease resembling tuberculosis. They concluded that this organism was the causative agent of the disease since the same organism was recovered from the patients before operation or autopsy. It was also present in the lesions of the surgical and autopsy material and was obtained from culture of the tissues. Furthermore, human tubercle bacilli were not recovered in any cultures from either case. In addition, one of the patients did not develop a positive tuberculin test.

Timpe and Runyon (1954) isolated acid-fast organisms differing from mammalian tuberculosis strains from 88 patients. Although some appeared to be initially associated with pulmonary disease in man, none was virulent for guinea pigs inoculated with 1 mg of the organism. They divided the organisms tentatively into three groups on the basis of colonial characteristics. Two of the groups (i and iii) were found to be virulent for mice while the other was not. The organisms displaying mouse virulence are characterized by repeated occurrence over long period of time in sputa from the same patients, none of whom were tuberculous. Seven of these strains were found directly in diseased human lung tissue. On the other hand, 17 strains of group ii showed no virulence for mice and were not as consistently associated with human disease. These organisms were found in lower concentration in original specimens, and a greater proportion of them was isolated from gastric washings. Furthermore, several were from patients harboring typical tubercle bacilli as well.



Prissick and Masson (1957) isolated ten strains of chromogenic unclassified acid-fast bacilli from tuberculoid lesions of cervical or facial lymphadenitis in children. The organisms were compared with some of the mycobacteria, including Mycobacterium tuberculosis, and Nocardia. On the basis of morphology, staining properties, cultural characteristics, pathogenicity, and allergic reactions in laboratory animals, they have been placed in the genus Mycobacterium. They proposed the name Mycobacterium scrofulaceum n. sp. to these strains owing to the fact of their isolation in pure culture from closed lesions, and their differences from other named species of the genus.

Buckle and Talhurst (1948) were able to isolate a pathogenic acid-fast organism from skin lesion in a series of six cases of ulceration of the skin among individuals in a rural area in Australia. Materials from ulcers infected rats and mice but not guinea pigs. In rats, characteristic lesions were produced including ascitis, cutaneous aedema, and ulceration; and the disease was transferable from rat to rat indefinitely. The organism, a strongly acid-fast mycobacterium, had been cultivated and its characteristics were described. They described the relationship of this disease to other mycobacterial infections, and concluded that the causal organism is distinct from any of the hitherto described mycobacteria. Norden and Linell (1951) recovered an acid-fast organism from skin lesions, usually localized to the elbows of patients who practiced swimming in a swimming pool in Orebro, Sweden. They also isolated the same organism from scrapings from the cemented wall of the pool and in samples of the water. The

pathogenic properties of the acid-fast bacilli isolated were studied experimentally in animals. The organisms were found to be only slightly pathogenic for guinea pigs, moderately pathogenic for rabbits, and potentially pathogenic for white mice and rats. They concluded that this isolated bacillus differs from the Australian bacilli as regards both growth on artificial media and their effect on experimental animals.

Anonymous mycobacteria were isolated from gastric contents of both healthy personnel and tuberculous individuals by Atwell and Prott (1960). Kending (1961) isolated acid-fast bacilli from gastric contents of children. Of 105 children with diagnosis of asymptomatic primary tuberculosis, 17 showed a positive gastric culture for acid-fast bacilli, eight of these were Mycobacterium tuberculosis, five were photochromogens, and four scotochromogens.

Since the unclassified mycobacteria are extremely heterogenous, there have been attempts to subdivide them into several more or less well defined groups. Early work on that field was done by Ruth E. Gordon (1937). She studied the cultural reactions of a collection of 252 unclassified acid-fast cultures from soil, plants, human and animal tissues, and secretions to find standards for the classification of these mycobacteria. Eighty per cent of the strains were readily separated into three groups on the basis of their ability to grow at different temperatures, colonial characteristics, and utilization of certain carbohydrates. Although no entirely satisfactory classification has as yet been established, several preliminary schemes have been proposed, and are presently in use. The most successful and useful

classification has been that of Runyan (1959), which consists of the following four groups:

Group i: Photochromogens. Greatly implicated in human disease. Pigment appears when cultures are exposed to light during growth period. Growth is slow at room temperature, or at 37C (Mycobacterium kansasii).

Group ii: Scotochromogens: Probably not pathogenic for humans although may be involved in cervical adinitis. Pigment is present regardless of the presence or absence of light during growth period. Yellow or orange pigmentation in the dark often becomes a deeper orange after exposure to light. Growth is the same as Group i.

Group iii: Non-photochromogens. "Battey Type". Greatly implicated in human disease. Very little or no pigment formation. Growth is same as Group i. Some are avian-like, some Nocardia-like.

Group iv: Rapid growers. Not usually implicated in human disease but, under certain circumstances, may be pathogenic. These are characterized by rapid growth, usually in three to four days, at both room temperature and 37C. Pigment is lacking in most strains. This classification is based primarily on colonial morphology, pigmentation, and rate of growth. Because variation in color and morphology of mycobacteria frequently occur, depending on the type of media used for their cultivation and other environmental conditions, it has been recommended (Gordon and Ryneerson, 1963) that more stable properties should be used for identifying these organisms.

A later classification by Marks and Trollope (1960) lumps the rapid growers (Runyan's group iv), but this makes too heterogenous a



group. It seems more useful to separate the rapid growers from the slower growing non-pathogens.

The distribution and frequency of isolated unclassified mycobacteria has not been determined very satisfactorily, but it is probably world-wide. In the United States, there is evidence that the various groups have a spotty distribution. In a comparative study on the distribution of unclassified mycobacteria in the United States, Gerszten et al. (1964) reported the following:

In Florida, Group iii-non-photochromogens accounts for almost three quarters of the total isolations of unclassified mycobacteria. The second most common group corresponded to the Group ii-scotochromogens in 13% of the cases. In Richmond, Virginia, the Group ii-scotochromogens predominated, being found in about four fifths of all cases. The second most common group, the Group iii-non-photochromogens made up 13% of the isolations. In Cleveland, Ohio, the predominating group resembles that in Virginia, but the second most common group was the Group i-photochromogens with 14% of the cases. Tarshis (1964) stated that in the north-central states and Texas, the Group i-photochromogens predominated.

Bojalil (1961), in Mexico, reported that the highest number of strains isolated was found to be the rapid growers followed in order of decreasing frequency by the scotochromogens, and finally by the non-photochromogens. Photochromogenic strains were not isolated from a total of 3,315 specimens. Furthermore, none of 15 additional strains of mycobacteria isolated from children was identified as photochromogens. The frequency of isolation of rapidly growing acid-fast bacilli

was the same in sputum and in gastric contents. On the other hand, scotochromogens as well as non-photochromogens were more frequently isolated from sputum than from gastric contents. Very few of the 175 strains of unclassified mycobacteria studied were apparently related to pathogenic processes.

In other parts of the world, even less is known about the distribution of human disease caused by unclassified mycobacteria. Group ii and Group iii organisms have been reported in Japan (Takeya et al., 1961), but the relationship of Group ii with human disease is debatable. Unlike the United States, no Group i-photochromogens have been implicated in human disease.

In Africa, a study of mycobacteria isolated from cervical lymph glands from patients in Kenya showed them to be all Mycobacterium tuberculosis (Sula et al., 1960), although in other areas this type of lesion often yields unclassified mycobacteria.

In Australia, Group iii and Group iv organisms were isolated from a number of tonsils removed from non-tuberculous children (Singer and Rodda, 1961). Reports of disease caused by unclassified mycobacteria in Southeast Asia are rare except in Indonesia where Tan et al. (1961) reported the isolation of 26 (2.6%) strains of anonymous mycobacteria from 993 human pathological specimens. Most of these were scotochromogens, but all groups were represented. A unique case of meningitis in Malaya due to a Group i-photochromogens has been published by Chan and Pathmanathan in 1961. There is also a considerable amount of indirect evidence for the existence of unclassified mycobacteria in human populations of many Asian countries.

A relatively high prevalence of low grade sensitivity or so-called non-specificity to the standard 5 TU tuberculin test has been reported (Edwards, Palmer and Magnus, 1953; Wijsmuller, 1959). This has been interpreted to indicate that the sensitivity reaction is invoked by exposure to an agent or agents other than Mycobacterium tuberculosis. Recent work also indicates that a high percentage of children in Kuala Lumpur react more strongly to PPD prepared from Group iii "Battey Type" strains, than to standard PPD-S (Chin, unpublished observations).

The problem of communicability of the unclassified mycobacteria from person to person is also equivocal. Based on some cutaneous hypersensitivity studies conducted in Texas and Louisiana, in which antigens prepared with the Mycobacterium kansasii strains were used, it was found that there was a higher degree of reactivity among household contacts of persons with disease due to these organisms than among the control groups (Ekman et al., 1961; and Chapman and Dewlett, 1961). In another study conducted in Georgia, however, in which antigens of the Battey bacilli were used, no disease could be detected in contacts of persons infected with these organisms (Crow et al., 1957). Another study of household contacts with children with mycobacterial lymphadenitis failed to reveal any individual with evidence of pulmonary disease (Chapman, 1962). That congenital disease may be caused by these bacteria has been pointed out recently (Beck et al., 1963).

## MATERIALS AND METHODS

### Sample Collection

Twenty-two soil samples were first collected from different places on campus, and near the campus of Kansas State University. Eleven other soil samples were later collected from eleven different areas, ten of these from different types of soil in the rural area around Manhattan, Kansas, and one from a known acid soil on campus.

### Detection of Acid-Fast Organisms

Two methods were tried to detect acid-fast bacilli in the soil samples.

The Buried Slide Technique. This method was carried out on fourteen samples from the first collection, and ten from the second collection. About 200 grams of soil were placed in a clean 250 ml beaker. The soil was moistened with distilled water, and a clean glass slide was half immersed vertically inside the soil. Beakers were then put in a desiccator, and left at room temperature from two to six weeks. Glass slides were then taken out carefully. One side of each slide was cleansed, and the other was partially freed from large mud particles. Slides were air dried, fixed by gentle heating, stained by the Ziel-Neelsen method, and examined microscopically. Observed acid-fast rods were reported.

Interface Concentration Method. Soil samples from the second collection only were examined by this method. Ten grams of each soil sample were put in a sterile screw-cap tube to which was added 10 ml of

a decontaminant containing 6 parts of 1.17% sodium hydroxide solution and 1 part sodium hypochlorite solution that contains 5-6% available chlorine, and 1 ml volatile hydrocarbon (heptane). The tubes were thoroughly shaken manually at intervals for 15-30 minutes, then centrifuged at about 3,000 r.p.m. for 20 minutes. A loopful from the film formed in the interface between the decontaminant and heptane was smeared on a clean glass slide, air dried, fixed by heat, stained by the Ziel-Neelsen method, and examined microscopically. Observed acid-fast rods were reported.

#### Isolation of Acid-Fast Organisms

Different methods and modifications were tried to recover acid-fast bacilli from soil.

Frey and Hagan Method. This method was carried out on 20 soil samples of the first collection. The procedure called for 60 ml portions of <sup>"</sup>Sohnsen's carbon-free medium in 200 ml Erlenmeyer flasks. The solution was composed of: potassium diphosphate, 0.5g; magnesium sulfate, 0.2g; ammonium chloride, 0.5g; sodium carbonate, a trace; and distilled water, 1,000 ml. The flasks were sterilized by autoclaving at 15 pound pressure for 30 minutes. After the medium had cooled, a sterile paraffin coated pebble, large enough so that it was only partially immersed in the fluid, was placed in each flask. The media were then inoculated with one gram of the soil samples, thoroughly shaken, and incubated at 47C. During the incubation some of the paraffin melted and formed a thin pellicle on the surface of the medium. Growth



was found within a week on the under side of the pellicle, on the paraffined surface of the pebble at the level of the surface of the medium, and also, in many cases, adhering to the sides of the flasks just above the surface of the medium. Smears from this growth stained by the Ziel-Neelsen method revealed no acid-fast rods. Flakes of paraffin from the enrichment flasks on which masses of growth were attached were streaked on a solid medium containing the same mineral salts of the liquid enrichment flasks, and incubated at 47C. Some colonies developed after about five days. Smears from these colonies stained by the Ziel-Neelsen method showed no acid-fast rods. Inocula from the colonies developed on the solid medium were streaked on Conn's medium that contains crystal violet in a concentration of 1:10,000 as an inhibiting agent. The formula of Conn's medium is as follows: agar, 15 g; glycerine, 10 ml; potassium diphosphate, 1 g; asparagin, 1 g; and distilled water, 1 liter. Plates were incubated at 47C, and no growth was detected.

Pour Plates + Naphthalene Atmosphere. Eight samples from the first collection were processed by this method. Serial dilutions of the soil sample in 1:100; 1:1,000; and 1:10,000 were inoculated in pour plates using a carbon-free mineral salt agar medium. After solidification of the medium, plates were placed in a desiccator in which a small beaker containing naphthalene was placed to provide the organisms with the required carbon. The desiccator was incubated at room temperature, and after varying periods of time tiny colonies in some plates were developed. Smears from these colonies stained by the Ziel-Neelsen method were negative for acid-fast rods.

Kubica Method. Ten soil samples from the second collection were used in this procedure. Two spatulas (5/8 by 3 inches) full of soil sample were placed in sterile screw-capped 50 ml centrifuge tubes. After the addition of 30 ml of sterile distilled water the tubes were shaken manually at intervals for 15-30 minutes to break up clumps, and suspend the microorganisms. The tubes were then allowed to stand until the supernatant fluid became homogeneous in appearance, at which time 10 ml portions were pipetted into screw-capped tubes containing equal volumes of a decontaminant composed of 6 volumes of 1.17% sodium hydroxide solution, and 1 volume of sodium hypochlorite solution which contained 5-6% available chlorine. Tubes were again shaken for 15-30 minutes, and then centrifuged in a regular centrifuge (about 3,000 r.p.m. for 15-20 minutes). The supernatant fluid, with the exception of about 1 ml was discarded, and the resuspended sediment, without neutralization, was inoculated onto Lowenstein-Jensen medium. All tubes were incubated at 37C. Any colonies growing on the media were smeared, stained by the Ziel-Neelsen method, and examined under the microscope. Only branched bacteria were detected, but not acid-fast organisms.

Modification for Kubica's Method. The same procedure of the previous method was performed except that instead of using the forementioned decontaminant, 10 ml of a 10% sodium triphosphate solution were added to 10 ml of the homogeneous supernatant fluid. The tubes were shaken well for 15-30 minutes, left at room temperature for 24 hours, and centrifuged. The supernatant fluid with the exception of about 1 ml was discarded, and the resuspended sediment without neutralization was inoculated onto Lowenstein-Jensen medium. Tubes

were incubated at 30C, and no acid-fast organisms were recovered because of the overwhelming contamination.

Soil + Decontaminant (sodium hydroxide-sodium hypochlorite solution) + Heptane. All the eleven soil samples from the second collection were processed by this method with duplication of soils. For isolation, 10 g of soil were placed in a sterile screw-capped tube, 10 ml of the sodium hydroxide-sodium hypochlorite solution, and 1 ml heptane were added. The tubes were thoroughly shaken by hand at intervals for 15-30 minutes, and divided into two groups. Tubes of one group were centrifuged directly after shaking; those of the other group were left for about 1 hour before centrifugation. A loopful from the pellicle formed in the interface between the decontaminant and the volatile hydrocarbon (heptane) was inoculated on Lowenstein-Jensen medium, and incubated at 30C. Any colonies growing on the media were smeared, stained by the Ziel-Neelsen method, and examined under the microscope. Any suspected contamination was further purified by treating a loopful of growth in about 1 ml of a 10% sodium triphosphate solution, shaken, and incubated at 30C for 24 hours. Tubes were either centrifuged, supernatant discarded, and sediment inoculated on Lowenstein-Jensen medium, or a loopful of the suspension was directly inoculated on Lowenstein-Jensen medium, and incubated at 30C. To check the purity of each culture, growth was streaked on nutrient agar plates to see if there is any difference in colonial morphology or microscopic characteristics. Pure cultures were then subcultured, and examined by a variety of physical and biochemical tests to determine their characteristics.



The Use of Mineral Salt-Mineral Oil Enrichment Method. Eight soil samples from the first collection, and ten samples from the second collection were processed by this method. Metal-capped test tubes containing about 7 ml carbon-free mineral salt medium, and 1 ml paraffin oil were sterilized by autoclaving at 15 pounds pressure for 30 minutes. After the media had cooled two sets of tubes were inoculated with serial dilutions of each soil sample from the first collection (1:100; 1:1,000; and 1:10,000). One set of tubes was incubated at 45C, and the other one at 30C. Tubes inoculated with the same serial dilutions of soil samples from the second collection were incubated at 30C. After about two weeks incubation growth in the form of a pellicle in the interface between the mineral salt and mineral oil was observed in tubes incubated at 30C only. Smears from some of these growths were stained by the Ziel-Neelsen method and revealed some acid-fast rods. For further purification of growth, a loopful of these pellicles was inoculated in tubes containing 1 ml of 10% sodium triphosphate solution, shaken, and incubated at 30C for 24 hours. A loopful of the suspension, or of the sediment left after centrifugation was inoculated on Lowenstein-Jensen media, and incubated at 30C. Colonies growing on the media were smeared, stained by the Ziel-Neelsen method, and examined under the microscope. To check the purity of cultures, growth was streaked on nutrient agar plates to see if there is any difference in colonial morphology or microscopic characteristics. Pure cultures were then subcultured, and examined by a variety of physical and biochemical tests to determine their characteristics.

## Identification of Acid-Fast Organisms

The staining characteristics, optimum growth temperature, rate of growth, and pigment production in the presence or absence of light were first determined for each culture. For further grouping other biochemical tests were performed.

Microscopic Examination. Smears were made from each pure culture, and stained by the Ziel-Neelsen method.

Temperature of growth. Groups of slants of Lowenstein-Jensen medium inoculated with each isolate were incubated at 28, 37, and 45C in incubators, and at 52C in a water bath, and examined for growth after 3 and 7 days incubation.

Survival at 60C. Lowenstein-Jensen media were inoculated with each organism and placed in a water bath at 60C for 4 hours. The tubes were cooled, and incubated at 28C for 14 days, and inspected for growth.

Rate of growth. Growth in inoculated Lowenstein-Jensen media with each organism was examined after 24, 48, and 72 hours incubation to determine the rate of growth of each organism.

Pigment Production. Two Lowenstein-Jensen slants were inoculated with each organism, one tube was wrapped in aluminum foil, the other was not. The tubes were incubated at 30C. When the unwrapped tube showed good growth, the foil was removed from the wrapped tube and its growth compared with the other one. The wrapped tube was exposed to light and examined the following day for any change in color.

The following biochemical tests were carried out for further identification of the isolated mycobacteria. The first five tests followed procedures used by the laboratories of the Wisconsin Public Health Department. Since certain of these procedures deviate slightly from published methods and since no published manual is available from the Wisconsin laboratories, the details of the methods are given completely.

The Arylsulfatase Test. Preparation of Media. One and three-tenths grams of dehydrated Bacto Dubos Broth Base was dissolved in 180 ml distilled water, sterilized in the autoclave for 15 minutes at 15 pounds pressure (121C), and cooled. When ready for use, 20 ml of Seitz filtered 5% bovine albumin, and 2 ml of 50% stock glucose solution, autoclaved at 121C for 15 minutes, were added to each 180 ml quantity of broth base. The medium was mixed thoroughly, and distributed aseptically in 16-20 mm diameter test tubes in 5 ml amounts. Tubes were incubated for 24 hours to test sterility.

Stock Solution. Two and six-tenths grams of tripotassium phenolphthaleine disulfate was dissolved in 50 ml distilled water to give a 0.08M stock solution which is then sterilized by Seitz filtration.

Working Solution. (1) A 0.001 M substrate solution was prepared by adding 2.5 ml of 0.08 M stock solution to 200 ml Tween-albumin broth and dispensed aseptically in 2 ml amounts in 15 x 125 mm screw-cap test tubes. This concentration was used for the three days test. (2) A 0.003 M substrate solution was prepared by adding 7.5 ml of the

0.08 M stock solution to 200 ml of Tween-albumin broth. Two ml of this solution were dispensed aseptically in 15 x 125 mm screw-cap test tubes. This concentration was used for the two weeks test.

Procedure. Tween-albumin broth tubes were inoculated with each culture and incubated at 37C for 7 days. Three tenths of a milliliter of a 7-day-old Tween-albumin broth culture was inoculated into each tube of both the 0.001 M, and 0.003 M substrate solutions, and incubated at 37C. After 3 days incubation of the 0.001 M cultures, 6 drops of a 2 N sodium carbonate solution were added to each tube with a capillary pipette, and change in color was reported immediately. After 2 weeks incubation of the 0.003 M cultures, 6 drops of a 2 N sodium carbonate solution were added to each tube with a capillary pipette, and any change in color was reported immediately. No color is negative, faint tinge of pink is questionable, and pale pink to deep red is positive.

Tween Degradation Test. The substrate was composed of M/15 phosphate buffer pH 7.0, 1,000 ml; Tween 80, 0.5 ml; and a 0.1% aqueous Neutral red solution, 2 ml. Four milliliters were dispensed in screw-cap tubes, and autoclaved at 121C for 15 minutes. A loopful of each culture was inoculated in each tube of substrate and incubated at 37C. Tubes were examined for any change in color of the substrate after 4 hours and once daily for 48 hours by comparing inoculated tubes with uninoculated control. Production of salmon pink to red color in supernatant within 48 hours after incubation is considered to be a positive reaction.

Nitrate Reduction Test. A loopful of each organism was suspended in a sterilized screw-cap test tube containing a few drops of sterile distilled water. Two ml amounts of M/100 solution sodium nitrate in M/15 phosphate buffer pH 7.0 which is composed of  $\text{NaNO}_3$ , 0.085 g;  $\text{KH}_2\text{PO}_4$ , 0.117 g;  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.485 g; and distilled water, 100 ml were added to each tube, shaken, and incubated at 37C for 2 hours. Then 1 drop of concentrated hydrochloric acid, 2 drops of 0.2% aqueous solution of sulfanilamide, and 2 drops of 0.1% aqueous N-naphthyethelenediamine dehydrochloride were added to each tube, and any change in color was reported. Any development of faint pink to red color is considered a positive test. Negative tests were confirmed by the addition of powdered zinc.

Urease Activity Test. One hundred ml M/100 phosphate buffer pH 6.8 was sterilized by autoclaving at 121C for 15 minutes. After cooling, 3 g of urea, and 1 ml of an aqueous 0.1% phenol red solution as an indicator were added. The medium was dispensed aseptically in 4 ml amounts in sterile screw-cap tubes, and incubated at 37C to check its sterility. One loopful of each organism was inoculated in each tube of medium and incubated at 37C for 3 days. Any change in color was then recorded. Change in color of phenol red indicated a positive result.

Niacin Test. One ml of sterile saline was added to culture slants of test organisms more than three weeks old. The cultures were then slanted for 15 minutes to allow the fluid to cover the growth. One-half milliliter of this aqueous extract was then transferred to a screw-cap tube to which 0.5 ml of 4% anilin in 95% ethyl alcohol, and 0.5 ml



of 10% aqueous solution of cyanogen bromide were added and mixed. Change in color was observed and reported. If niacin is present a yellow color appears almost immediately through the solution, but no color indicates the absence of niacin.

Regular Catalase Test. About 1 ml of 3% hydrogen peroxide was added to each culture slant and observed for the formation of oxygen bubbles.

Thioglycollate Test. Dehydrated Bacto Thioglycollate medium was used. Screw-cap tubes containing 5 ml amounts of sterile rehydrated thioglycollate medium were inoculated with one loopful of each test organism which had grown for approximately 7 days in Tween-albumin liquid medium or with the tip of a platinum wire which had been dipped into the bacterial masses of similar aged organisms growing on Lowenstein-Jensen medium. The inoculated media were incubated at 37C for several days, and the tubes were examined daily for detection of growth. Thioglycollate-positive strains grow in the medium, whereas negative strains do not (Tarshis, 1961).

Peroxidase Test. One ml of a 2% catechol solution, and 0.5 ml of a 1% aqueous solution of hydrogen peroxide were placed into each of a series of screw-cap tubes and inoculated with a loopful of growth of each culture of the test organisms. The tubes were then incubated for 24 hours at room temperature. The results were read after 10 minutes, and again after 1, 12, and 24 hours, respectively. Peroxidase-positive strains turn brown within 10 minutes, darken and become black within 1 to 24 hours. Negative strains remain unchanged (Tarshis, 1961).

Neutral Red Cytochemical Test. Five ml of 50% methanol were placed into each of a series of screw-cap tubes, and were inoculated with a loopful of growth from each of the cultures. The tubes were incubated for 1 hour at 37C then centrifuged to pack the organisms, and the alcohol was decanted. Five ml of the alcohol were again added to each tube, the organisms were resuspended by shaking the tubes, incubated for one hour at 37C, centrifuged, and the alcohol decanted. Five ml of alkaline-barbiturate buffer composed of sodium barbitol, 10 g; sodium chloride, 5.0 g; and distilled water, 100 ml and 0.2 ml of a 0.05% aqueous solution of neutral red were added to each tube, the contents were shaken, and the tubes were incubated for one hour at 37C. The tubes were shaken thoroughly every 15 minutes, and the results were read after the period of incubation. Neutral red positive strains exhibit varying degrees of pink or red staining of the organisms. Negative strains remain unchanged (Tarshis, 1961).

Acid Production From Carbohydrates. Amounts (0.5 ml) of a 10% aqueous solution of the carbohydrate to be tested, sterilized by autoclaving, were added aseptically to tubes containing 5 ml of sterile inorganic nitrogen base, a modification of one proposed by Ayers, Rupp, and Johnson (1919). The inorganic nitrogen base had the following composition:  $(\text{NH}_4)_2\text{HPO}_4$ , 1 g; KCl, 0.2 g;  $\text{MgSO}_4$ , 0.2 g; Agar, 15 g; distilled water, 1,000 ml. The pH of the medium was adjusted to 7.0 before the addition of 15 ml of a 0.04% aqueous solution of bromcresol purple (Gordon and Smith, 1955). Media were inoculated with each organism, and cultures on each carbohydrate

medium were observed for acid production as revealed by the change of the indicator to yellow after 7 and 28 days of incubation at 28C. The following carbohydrates were tested: glucose, mannose, fructose, galactose, L-arabinose, xylose, rhamnose, lactose, maltose, sucrose, trehalose, melibiose, raffinose, mannitol, sorbitol, m-inositol, erythritol, and dulcitol.

Utilization of Organic Acids as Carbon Sources. The cultures were inoculated on slants of modifications of Koser's citrate agar medium (1924). The medium is composed of: NaCl, 1 g;  $MgSO_4$ , 0.2 g;  $(NH_4)_2HPO_4$ , 1 g;  $KH_2PO_4$ , 0.5 g; sodium citrate, or sodium benzoate, or sodium succinate, or sodium-potassium tartrate, 2 g; agar, 15 g; and distilled water, 1,000 ml. The pH of each medium was adjusted to 7.0 before the addition of 20 ml of a 0.04% solution of phenol red. The tubes were incubated at 28C, and the use of the organic acid was indicated by the alkaline color of the phenol red after 7 and 28 days incubation.



## RESULTS

Microscopic examination of slides prepared by the buried slide technique, and the interface concentration method showed a variety of acid-fast structures. Only acid-fast rods were considered to be positive for detection of acid-fast bacilli. Table 1 shows the percentage of positive slides prepared by both methods.

Table 1. Percent of slides showing acid-fast rods prepared by the buried slide technique and the interface concentration method.

Collection	Buried slide technique			Interface concentration method		
	No. of	No. of		No. of	No. of	
	samples	positives	Percent	samples	positives	Percent
	: tested :	:	:	: tested :	:	:
First collection	14	5	35.7	None		
Second collection	10	3	33.3	10	5	50

## Isolation of Acid-Fast Organisms

No acid-fast organisms were isolated by any of the following methods: Frey and Hagan method, the mineral salt agar pour plate + naphthalene atmosphere, Kubica method, and its modification. Acid-fast organisms were recovered by the procedures of soil + decontaminant + heptane in both direct centrifugation, and centrifugation one hour after shaking, and mineral salt mineral oil enrichment.

Out of eight soil samples from the first collection, four cultures were isolated by the mineral salt-mineral oil enrichment. Two of these cultures were chromogenic in both dark and light, one non-chromogenic, and one developed a pinkish discoloration when the culture became old.

From the eleven soil samples of the second collection, fourteen cultures were recovered. Three of these cultures were isolated by the mineral salt-mineral oil enrichment. These were chromogenic in both dark and light. The other cultures were non-chromogenic, and isolated by the soil + decontaminant + heptane method. Five of these cultures were recovered from the interface formed by centrifugation directly after shaking, the other six from the interface formed by centrifugation an hour after shaking. Table 2 shows the number of cultures isolated from the soil samples processed by both methods.

#### Identification of Isolated Microorganisms

Microscopic and Staining Characteristics. The isolated microorganisms were variable microscopically both in morphology and staining characteristics. Acid-fastness in the majority of isolated organisms was observed in 80-100% of the cells. In culture No. S-B acid-fastness was less than 50%.

Effect of Temperature on Growth. All cultures grew well at 28 and 37C. Incubation at 45C was destructive to some organisms and inhibitory to other ones. After 7 days incubation growth was detected in only 3 of a total of 18 cultures. Non-chromogenic culture No. 9A

Table 2. Number of cultures isolated from soil samples by the Soil + Decontaminant + Heptane; and the Mineral Salt-Mineral Oil Enrichment methods.

Soil sample	Method of Isolation							
	Soil + Decontaminant + Heptane				Mineral salt-Mineral oil enrichment			
	Direct centrifugation		Centrifugation after 1 hour					
	Chromogenic	Non-chromogenic	Chromogenic	Non-chromogenic	Chromogenic	Non-chromogenic	Total	
4A	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	1	none	1	
7A	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	none	none	none	
14	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	none	1	1	
15	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	none	none	none	
18	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	none	none	none	
20	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	1	none	1	
S-B	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	none	1*	1	
a-a	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	..... NOT DONE .....	none	none	none	
1	none	none	none	none	none	none	none	
2	none	none	none	none	none	none	none	
3	none	none	none	none	none	none	none	
4	none	none	none	none	none	none	none	
5	none	1	none	1	none	none	2	
6	none	none	none	1	1	none	2	
7	none	1	none	none	none	none	1	
8	none	none	none	1	none	none	1	
9	none	2	none	1	1	none	4	
10	none	1	none	1	1	none	3	
11	..... NOT DONE .....	..... NOT DONE .....	none	1	..... NOT DONE .....	..... NOT DONE .....	1	
TOTAL	none	5	none	6	5	2	18	

\*Becomes pinkish in color when old.

showed good growth, culture No. 7 scant growth, and culture No. 9 (1 hr.) was questionable. After the required incubation period, the tubes were incubated at room temperature. In five to eight days, growth became luxuriant in culture No. 7, and visible growth appeared in non-chromogenic cultures Nos. 9 (1 hr.), 14, 9B, 10, 6 (1 hr.), 10 (1 hr.), and 5. None grew at 52C or survived after 4 hours at 60C.

Rate of Growth. All cultures grew rapidly, showing visible growth in one to three days.

Pigment Production. Pigments produced by chromogenic organisms were not influenced by light. They were developed both in dark and light. Chromogenic cultures No. 4A and 9 were orange red, and cultures No. 20, 10, and 6 were yellow orange. All non-chromogenic cultures were pale straw, and culture No. S-B developed a pinkish discoloration when old.

Physiological Tests. The physiological characteristics of the isolated organisms are listed in tables 3 and 4.

Arylsulfatase Test. In the 3-day test, non-chromogenic cultures No. S-B, 7, 9A, 9B, and 9 (1 hr.) gave a 4+, and culture No. 11 (1 hr.) gave a 1+. According to Kubica's data (1961), cultures giving a 4+ positive test may represent the presence of Mycobacterium fortuitum. In the 2-week test, culture No. 4A was negative, chromogenic cultures No. 6 and 9 were questionable, culture No. 20 was 2+, chromogenic culture No. 10 and non-chromogenic culture No. 10 gave a 3+, culture No. 8 (1 hr.) a 4+, and non-chromogenic cultures No. 14, S-B, 5, 7, 9A, 9B, 6 (1 hr.), 9 (1 hr.), 10 (1 hr.), 5 (1 hr.), and 11 (1 hr.) gave a 5+ reading.

Table 3. Morphological and physiological characteristics of the isolated, rapidly growing, unclassified mycobacteria.

Test	Chromogenic isolates										Non-chromogenic isolates								
	4A	20	6	9	10	14	S-B	5	7	9A	9B	10	6	9	10	5	8	11	
													(1 hr.)	(1 hr.)	(1 hr.)	(1 hr.)	(1 hr.)	(1 hr.)	
Pigment in Lowenstein-Jensen	orange red	yellow orange	yellow orange	orange red	yellow orange	pale straw	pale* straw	pale straw	pale straw	pale straw	pale straw	pale straw	pale straw	pale straw	pale straw	pale straw	pale straw	pale straw	
Acid-fastness	4+	4+	4+	4+	4+	5+	2+	3+	5+	5+	5+	5+	5+	5+	5+	3+	3+	3+	
Temp. of growth																			
28C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
37C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
45C	-	-	-	-	-	-!	-	-!	+	+	-!	-!	-!	+	-!	-	-	-	
52C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Survival after 4 hr at 60C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Growth rate																			
Rapid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Slow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Arylsulfatase 3-days	-	-	-	-	-	-	4+	-	4+	4+	4+	-	-	4+	-	-	-	1+	
2-weeks	-	2+	+	+	3+	5+	5+	5+	5+	5+	5+	3+	5+	5+	5+	5+	4+	5+	
Tween degradation	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	
Nitrate reduction	-	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	-	+	
Urease activity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Niacin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Regular catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Peroxidase	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	
Neutral red cytochemical	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	

\* Becomes pinkish when old.

! Cultures, its growth is inhibited at 45C, but survive and grow 5-8 days later at room temperature.

\*\* Time elapsed between shaking of the tubes and centrifugation.

Table 4. Acid production from carbohydrates, and utilization of organic acids by the isolated rapidly growing unclassified mycobacteria.

Test	Chromogenic isolates					Non-chromogenic isolates												
	4A	20	6	9	10	14	S-B	5	7	9A	9B	10	6	9	10	5	8	11
	:	:	:	:	:	:	:	:	:	:	:	:	(1 hr.)*	(1 hr.)	(1 hr.)	(1 hr.)	(1 hr.)	(1 hr.)
Acid production from carbohydr.																		
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M-inositol	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+	+
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of Benzoate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Citrate	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tartrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\* Time elapsed between shaking of tubes and centrifugation.



Tween Degradation Test. All chromogenic organisms and culture No. S-B gave a positive result. Non-chromogenic culture No. 14 was questionable. The rest of the isolates were negative.

Nitrate Reduction Test. Chromogenic cultures No. 4A, and 9, and non-chromogenic cultures No. S-B, 5, 5 (1 hr.), and 8 (1 hr.) were negative. The rest of the cultures were positive.

Urease Activity Test. All isolates were positive.

Niacin Test. All cultures were negative.

Regular Catalase Test. All cultures showed powerful catalase reaction.

Thioglycollate Test. This test was performed several times, but no reliable results were available.

Peroxidase Test. All chromogenic cultures were negative, isolate No. S-B was questionable, and the rest of cultures were positive.

Neutral Red Cytochemical Test. All chromogenic cultures, and non-chromogenic cultures No. S-B, 5, 5 (1 hr.), 8 (1 hr.), and 11 (1 hr.) were negative. The rest of the cultures were positive.

Acid Production From Carbohydrates. Chromogenic cultures were more active than non-chromogenic ones. They ferment glucose, mannose, fructose, L-arabinose, xylose, trehalose, mannitol, and m-inositol. Chromogenic culture No. 6 does not ferment galactose and rhamnose, and pigmented cultures No. 20 and 10 do not ferment rhamnose. Lactose, sucrose, maltose, melibiose, raffinose, sorbitol, erythritol, and dulcitol were not utilized by the chromogenic isolates. Non-chromogenic organisms were less active than chromogenic ones. All of them ferment

glucose and fructose. Only culture No. S-B failed to ferment mannose. Trehalose is utilized by all non-chromogenic organisms except cultures No. 14 and 10 (1 hr.). Mannitol is utilized by all non-chromogens except cultures No. 7, 9A, 9B, and 9 (1 hr.). M-inositol is utilized by non-chromogenic cultures No. 5, 5 (1 hr.), 8 (1 hr.), and 11 (1 hr.).

Utilization of Organic Acids. Chromogens and non-chromogens utilized succinate as a carbon source. None of them utilized tartrate. Benzoate was not utilized by any of the two groups of organisms except the non-chromogenic culture No. 11 (1 hr.). Citrate was utilized by three of the pigmented cultures, No. 4A, 6 and 9, and by two of the non-pigmented ones, No. S-B and 8 (1 hr.).

#### DISCUSSION

From the data presented, it is obvious that microscopic detection of acid-fast bacilli in soil is possible by simple methods, although other acid-fast structures may be present. The interface concentration method for detecting acid-fast rods seems to be superior to the buried slide technique, since a larger percentage of acid-fast rods was detected by this method, and because it is a simple and rapid procedure. Not all samples showing positive smears yielded acid-fast organisms on isolation. The methods adopted in this work proved efficient only for isolating rapid-growing acid-fast bacilli, and one can not ignore the possibility of having slow-growing unclassified mycobacteria in soil.



The question raised on the inability of isolating acid-fast bacilli by Frey and Hagan method can be resolved by the fact that the growth of the majority of the isolates was either destroyed or inhibited on incubation at 45C. These results agree with the data presented in the work done by Gordon and Hagan (1937). Cultivation in a naphthalene atmosphere did not prove satisfactory since no acid-fast forms were detected by this procedure. It was surprising that no acid-fast organisms could be isolated by the Kubica method (1963). It seems likely that most of the microorganisms suspended in the homogenized soil suspension did not withstand the drastic effect of the decontaminating solution, and that branching bacteria or conidia escaped this effect and survived on culturing onto Lowenstein-Jensen medium.

The theory used in the phase separation procedure for detecting and recovery of acid-fast bacilli from soil is based on the fact that acid-fast organisms possess a hydrophobic and lipophilic nature (Chodosh and Gray, 1960). On centrifugation these organisms are concentrated in a film in the interface between the decontaminant and the hydrocarbon used. A volatile hydrocarbon, heptane, was selected to overcome the problem of drying and fixing the smear before staining. The method, despite its incapability in the recovery of slow-growing acid-fast bacteria, is a satisfactory one for isolating rapid growers. It is obvious that only rapid-growing acid-fast organisms were recovered by this method due to the overgrowth by other forms on culturing. The mineral salt-mineral oil enrichment served in the concentration

of some acid-fast bacilli in a film of growth in the interface between the mineral salt and mineral oil, i.e., paraffin. One of the most interesting observations is that all the chromogenic cultures were isolated only by the mineral salt-mineral oil enrichment method. The soil + decontaminant + hydrocarbon method seems to be superior to the mineral salt-mineral oil enrichment method in that in the same soil collection, more cultures were isolated by the former method than by the latter. Only non-chromogenic acid-fast bacilli were recovered by the soil + decontaminant + hydrocarbon, while both chromogenic and non-chromogenic acid-fast organisms were isolated by the mineral salt-mineral oil enrichment method. In the soil + decontaminant + hydrocarbon method, it seems obvious that there was an insignificant difference between centrifuging the tubes directly after shaking or an hour later. The method may be improved by allowing the decontaminating solution to stand with the soil sample for a longer period of time before centrifugation.

All isolates grown on Lowenstein-Jensen medium showed acid-fastness on staining by the Ziel-Neelsen method. The percentage of cells which were acid-fast ranged from 80-100% in the majority of cultures, and 50% or less in a few of isolates. The cells differ morphologically from cocco-bacillus, short rods, to long and slender non-filamentous ones. Some of the cells show beads. All isolates are rapid growers, and grow well at 28C and 37C. Incubation at 45C is destructive to some organisms, and inhibitory to others that resume growing when incubated at room temperature for several days. None of

the cultures grow at 52C or survive after 4 hours incubation at 60C. The pigment production among chromogenic cultures was not influenced by light.

Among the physiological tests employed in this work, the 3-day arylsulfatase test, and the utilization of carbohydrates and organic acids tests were of some help in identifying the organisms, although no accurate determination of species was achieved.

According to the scheme established by Bojalil et al. (1962) for the classification of pigmented, rapid-growing unclassified mycobacteria, the chromogenic cultures isolated in this work fit to a great extent the group termed "irregular branch". The organisms No. 20 and 10 may be of the same strain, since they are similar in their reaction with rhamnose and citrate, and in other morphological and physiological characteristics. The organisms No. 4A and 9 may be of another strain in the same group as they behaved the same way in their utilization of rhamnose and citrate. Moreover, they showed other similarities in both morphological and physiological characteristics. Organism No. 6 behaved in the border line between the two forementioned strains, because it did not ferment rhamnose as in the first strain, i.e., cultures No. 20 and 10, but utilized citrate as the second strain did, i.e., cultures No. 4A and 9.

Regarding the utilization of carbohydrates and organic acids, among the non-chromogenic isolates, organisms No. S-B and 8 (1 hr.) showed some similarities to Mycobacterium fortuitum. They differed from it in that culture No. S-B did not ferment mannose, and culture No. 8 (1 hr.) fermented m-inositol. Moreover, organism No. 8 (1 hr.)

was negative to the 3-day arylsulfatase test, which is considered indicative for Mycobacterium fortuitum, hence, organism No. 8 (1 hr.) should be excluded from that assumption. It seems that non-chromogenic organisms No. 7, 9A, 9B, and 9 (1 hr.) were the same, since they reacted the same in both the utilization of carbohydrates and organic acids. Furthermore, this group of organisms were positive to the 3-day arylsulfatase test which is indicative for Mycobacterium fortuitum, but they did not utilize citrate. These four cultures may represent one strain of Mycobacterium fortuitum, and culture No. S-B may represent another strain of the same species. Unless a pathogenicity test should be performed on such isolates, no definite determination could be established.

Bojalil et al. (1962), in their study on rapidly-growing non-chromogenic acid-fast bacilli suggested a name for a new species, Mycobacterium runyonii sp. nov., which was negative for both acid production from fructose and trehalose, and utilization of citrate. In this work, if the utilization of fructose and mannitol by the isolates No. 14 and 10 (1 hr.), which seem to be identical, and the utilization of fructose and trehalose by the non-pigmented isolates No. 10 and 6 (1 hr.), which seem to be identical also, are disregarded, these two groups of organisms could represent two different strains of Mycobacterium runyonii sp. nov.

Non-chromogenic cultures No. 5, 5 (1 hr.), 8 (1 hr.), and 11 (1 hr.) are similar in their acid production from carbohydrates but differ slightly in their utilization of organic acids. This group of organisms may represent another Mycobacterium species.

## SUMMARY

Nineteen soil samples were investigated using six methods for detection and isolation of acid-fast bacteria from soil. Acid-fast bacilli could be detected in soil by simple laboratory methods. Six methods and modifications were carried out to isolate acid-fast organisms from soil. Two simple techniques were devised by which 18 rapid-growing unclassified mycobacteria were recovered in pure culture from soil. Five cultures were chromogenic, and thirteen were non-chromogenic. The chromogenic acid-fast isolates fit the description of the so-called "irregular branch" reported by Bojalil et al. (1962). Three different stains were suggested to represent this new species. The non-chromogenic isolates were heterogenous in their physiological characteristics. These were grouped into three categories:

1. Five cultures are suspected to represent two different strains of Mycobacterium fortuitum. No definite determination can be established unless pathogenicity studies could be performed.
2. Four isolates are suggested to represent two different strains of a new species termed Mycobacterium runyonii sp. nov. by Bojalil et al. (1962).
3. Four isolates are suggested to represent a Mycobacterium sp.

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METHODS FOR THE ISOLATION OF MYCOBACTERIA FROM THE SOIL

by

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AN ABSTRACT OF A MASTER'S THESIS

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Many false positive tuberculin reactions are believed to be due to sensitivity caused by some members of the so-called unclassified mycobacteria. A variety of these anonymous mycobacteria were isolated from different sources including soil, grass, leaves, water, and animal excreta. The lack of communicability of diseases caused by the unclassified acid-fast bacilli leads one to postulate some source in nature, other than man, as a reservoir for these microbes. The high rate of human infection in the southeastern United States would also suggest some very common "natural reservoir" to which a large percentage of the population is exposed. It was suggested that the soil could be the probable source for unclassified mycobacteria. Since then, many attempts were done to improve the methods of recovery of acid-fast bacilli from soil in pure culture.

Nineteen soil samples were processed for the detection and isolation of acid-fast bacilli from soil. Acid-fast rods could be detected in soil by simple laboratory methods. Different methods and modifications were carried out to isolate acid-fast microorganisms from soil. Two simple techniques were devised by which eighteen rapid-growing unclassified mycobacteria were recovered in pure culture from soil.

1. A phase separation procedure in which soil samples were processed in the presence of a decontaminating solution, and a volatile hydrocarbon. The theory of this procedure is based on the fact that acid-fast organisms possess a hydrophobic and lipophilic nature.

2. A mineral salt-mineral oil enrichment procedure in which some acid-fast organisms were concentrated in a film of growth in the interface between the two fluids.

Of the eighteen isolates, five cultures were chromogenic and thirteen were non-chromogenic. The chromogenic cultures fitted the description of the so-called "irregular branch" reported by Bojalil et al. (1962). Three different strains were suggested to represent this new species. The non-chromogenic cultures were heterogenous in their physiological characteristics. These were grouped into three categories:

1. Five cultures were suspected to represent two different strains of Mycobacterium fortuitum. No definite determination can be established unless pathogenicity studies would be performed.

2. Four isolates were suggested to represent two different strains of a new species termed "Mycobacterium runyonii" sp. nov. by Bojalil et al. (1962).

3. Four cultures were suggested to represent a Mycobacterium sp.