TITLE

BACTERIOLOGICAL AND DRUG SENSITIVITY STUDIES ON MYCOBACTERIA ISOLATED FROM TUBERCULOSIS PATIENTS AND THEIR CLOSE CONTACTS IN AHMADU BELLO UNIVERSITY TEACHING HOSPITAL, ZAR1A.

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C E R T I F I C A T I O N

This thesis titled "Bacteriological and Drug Sensitivity Studies on Mycobacteria Isolated From Tuberculosis Patients and Their Close Contact in Ahmadu Bello University Teaching Hospital, Zaria"

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D E C L A R A T I O N

This is to certify that I carried out the work reported in this thesis at Tuberculosis annexe, Ahmadu Bello University Teaching Hospital and the laboratories of the Department of Microbiology, Faculty of Science and Department of Pathology and Microbiology, Faculty of Veterinary Medicine (FVM), Ahmadu Bello University, Zaria, under the supervisions of Professors P. B. Addo, A. A. Ahmed and Dr. (Mrs) J. U. Okpapi.

This thesis is an original work carried out by myself and no part of it has been presented elsewhere for a degree or diploma. The work of other investigators is acknowledged and referred to in this presentation.

Ahmadu Bello University, Zaria
May, 1991

IBRAHIM, K.

D E D I C A T I O N

In revered memory of my dad,
Late M. Muhammad Baba-Nnatayi

A C K N O W L E D G E M E N T

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ABSTRACT

Tuberculosis is prevalent in all the regions of the world and it affects all parts of the body. It is one of the major public health problems in the developing countries. Fifty four species of Mycobacterium are currently recognized, 25 are either obligate or opportunistic pathogens. The diseases caused by some of these species closely resemble those caused by M. tuberculosis. Their speciation and differenciation from Nocardia which is slightly acid-fast are done by cultural and biochemical techniques. Various anti-tuberculosis drugs have been developed and when given in optimal combination, can cure almost every patient suffering from tuberculosis. However, drug resistance in tuberculosis has been a serious problem throughout the world especially in areas where the disease prevalence is high. It is against this background, that this investigation was carried out to speciate mycobacterial isolates and identify other organisms involved in pulmonary disease that mimic tuberculosis. In addition, the relationship between tuberculin skin reaction and laboratory techniques in house hold contacts of tuberculosis patients and the sensitivity patterns of the mycobacterial isolates to the 'WHO model list' of anti-tuberculosis drugs were investigated.

Three hundred various clinical samples were collected from suspected tuberculosis patients whose ages range between 11 to 68 years. The samples consisted of 229 sputa, 26 urine, 6 peritoneal and 36 pleural fluids, 3 pus, 1 bone marrow and 3 lymph nodes.

The samples were decontaminated employing the Petroff's modified method of N-acetyl-L-cysteine - Sodium hydroxide, and concentrated by spinning at 3,000 revolutions per minute for 20 minutes. Smears prepared from the sediment were stained by Ziehl-Neelsen, Kinyoun modified and Gram's techniques and were observed under the microscope.

Using sterile Pasteur Pipette, Lowenstein-Jensen and Oleic acid-dextrose - catalase media were inoculated. Samples from the same patients were also examined for the presence of Nocardia species by inoculating reconstituted samples into Sabouraud dextrose and blood agar plates. Tentative identification of the mycobacterial isolates was by the determination of speed of growth, chromogenicity and biochemical tests which include niacin production, nitrate reduction, semiquantitative catalase production, Tween hydrolysis, arylsulphatase production and heat-stable catalase tests. Nocardia was identified employing the Kinyoun modified cold-staining method, Gram's staining, pellicle formation in thioglycollate broth, utilization of paraffin and hydrolysis of casein.

One hundred and fifty sputa were collected from contacts of tuberculosis patients who had no BCG scar and who were Mantoux test positive. The samples were examined by direct microscopy and culture for the presence of acid-fast bacilli. A total of one hundred and thirty five mycobacterial isolates from tuberculosis patients and their contacts were tested for drug sensitivity against the 'WHO model list' of anti-tuberculosis drugs.

The isolates consisted of 86 M. tuberculosis, 3 M. bovis, 18 M. kansasii, 9 M. avium complex, 9 M. fortuitum, 4 M. smegmatis and 6 unidentified isolates.

One hundred and forty two (47.33%) of the samples from the suspected tuberculosis patients were positive for acid-fast bacilli by the direct microscopic method, while 134 (44.66%) isolates were recovered culturally. Tentative identification of the isolates showed 75 M. tuberculosis, 3 M. bovis, 17 M. kansasii, 9 M. avium complex, 9 M. fortuitum, 4 M. smegmatis and 17 unidentified isolates. Eleven of the unidentified isolates were identified as Nocardia asteroides, while the remaining six were unidentifiable.

Twelve patients were confirmed to habour acid-fast bacilli by direct microscopic and cultural examinations among the 150 close contacts of tuberculosis patients who were positive for tuberculin skin test. This represents 8.0 per cent. Mantoux reaction or its size bore no relationship with direct microscopic and cultural examinations.

Primary resistance of M. tuberculosis was most frequent to isoniazid (18.66%) and streptomycin (13.33%), and for thiacetazone, ethambutol, pyrazinamide and rifampicin, it was 9.33%, 6.66%, 5.33% and 4.00% respectively. One isolate of M. tuberculosis showed multiple resistance to isoniazid, streptomycin, pyrazinamide and thiacetazone and four isolates showed multiple resistance to two of the drugs. Acquired resistance of M. tuberculosis was highest to isoniazid (27.7%), streptomycin (18.18%) and thiacetazone (18.18%). For each of rifampicin, pyrazinamide and ethambutol it was 9.09%. Multiple drug resistance in patients with history of previous therapy was also observed. One patient showed multiple resistance to all the drugs except ethambutol and another patient showed resistance to isoniazid, streptomycin and thiacetazone.

Both primary and acquired resistances were also observed in the other species. Generally, the rates of primary drug resistance shown by a particular specie of Mycobacterium were lower than for acquired resistance to any of the drugs.

Petroff's modified method of decontamination of clinical samples for the study of mycobacteria was found to be convenient, easy and gave very good decontaminative effect on other microorganisms of the samples in this study. The decontamination efficiency of this reagent range between 86.66 per cent for urine samples and 100 per cent for other body fluids. The isolation of atypical mycobacteria and Nocardia asteroides calls for caution in the interpretation of acid-fast bacilli seen on direct smear because, from this work it is observed that not all acid-fast bacilli seen by direct microscopy are M. tuberculosis.

It also calls for care in the formulation of chemotherapeutic regimen for the individual tuberculosis patient in our hospitals since antituberculosis drugs do not affect Nocardia. The incidences of both primary and acquired resistances also require additional care in formulating chemotherapy programmes by the clinicians. In developing countries a good history of previous contact with the organism and drugs is very important in order to detect defaulters.

There is also the need to monitor the contacts of tuberculosis patients employing at least the direct microscopy technique to determine their infectious status.

Since the laboratories in which this work was carried out are the first and second types of laboratories based on the classification of Mycobacteriology Laboratories by Centres for Disease Control, Atlanta, Georgia, U. S. A., the isolates are being kept until they can be confirmed and the unidentifiable isolates are identified. Meanwhile, they will be subcultured at regular intervals to keep them alive.

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CHAPTER ONE

I N T R O D U C T I O N

1. INTRODUCTION

Tuberculosis is prevalent in all parts of the world tropical, subtropical and colder regions (Mudaliar, 1981). It affects all parts of the body including the brain and the meninges, the bones, the joints, abdominal and thoracic viscera (Davidson and Horowitz, 1970, Lenzine et al. 1977, Wolinsky, 1979, ATS, 1981). Today, it is considered to be one of the major public health problems in developing countries of the world (Bordia, 1981, Grange, 1989).

The genus Mycobaterium, the etiologic agent of mycobacteriosis is ubiquitous in soil, in water and in air, and the disease is a world wide problem afflicting all races (Lincolyn and Gilbert 1972, Siddiqi et al. 1976) Its prevalence and severity prompted the studies of Koch and others on the etiology, diagnosis, and prevention in the late 1800. In the first half of the twentieth century, other mycobateria isolates from patients with pulmonary disease were often incompletely described or discarded (Good, 1985). A recent advance in the knowledge of clinical mycobacteriology was the recognition that acid - fast bacilli other than human or bovine tubercle bacilli could cause significant clinical disease in man (Farer et al. 1979; wolinsky, 1979; Good, 1985).

Currently, 54 species of Mycobacterium are recognized of which about 25 are either obligate or opportunistic pathogens (Good, 1985). The disease entities caused by some of these species closely resemble those caused by M tuberculosis, and in fact have pathologic, and to some extent clinical similarities to tuberculosis, though there are distinct differences in virulence, treatment and prognosis (Xalabarder, 1961; Goodwin, 1975). Diagnosis of the disease is based on sputum examination for acid-fast bacilli or X-ray. These have made it impossible to differentiate among the various species of Mycobacterium or even Nocardia.

The important basis for differentiation between the typical and atypical mycobaterial infections is therefore a biological one requiring the service of a laboratory diagnostician to establish the precise mycobacterial agent.

Despite the huge amount of research, there is no single, reliable and rapid immunological or chemical test for the diagnosis of active tuberculosis (Grange, 1989). The problem posed by diagnostic difficulties in both individual care and in control programmes are enormous. A rapid diagnosis is therefore crucial yet so difficult to achieve. The only sure way of diagnosing tuberculosis at present is to find the tubercle bacillus either by direct microscopy or culture technique or both (Mitchison, 1972, Grange, 1989). Unfortunately, cultural methods are slow and insensitive enough in multibacillary pulmonary tuberculosis and the difficulties are greatly magnified in the case of paucibacillary pulmonary and nonpulmonary disease.

Historically, the first immunodiagnostic test was the tuberculin test (Lunn, 1983). The problem of this in respect to diagnosis are enormous: the inability to distinguish active tuberculosis from past sensitization by tubercle bacilli, BCG or environmental mycobacteria (Ten Dam and Hitze, 1980). In view of the problems encountered with tuberculin test and with other tests for cell-mediated immunity, much attention has been directd towards the serodiagnosis of tuberculosis (Reggiardo and Vazquez, 1981; Daniel and Debanne 1987; Caplin et al. 1989; Grange, 1989). These tests are aimed at competing with sputum microscopy with respect to sensitivity, specificity, ease of organisation and quality control, acceptibility to patients, technical simplicity and cost (Grange, 1989). Accordingly to date, no test has satisfied these very rigorous criteria for widespread clinical usage.

One of the major medical advances of the twentieth century has been the development of anti-tuberculosis drugs.

Citron (1982) and Mitchison (1984) separately reported that when given in optimal combination, these drugs will cure almost every patient suffering from tuberculosis; and that the application of chemotherapy, together with improved social conditions, has resulted in a spectacular decline of tuberculosis in technically advanced countries.

However, drug resistance in tuberculosis is a serious problem throughout the world especially in those areas where the disease prevalence is high and in communities where there is no organised anti-tuberculosis therapy programme and where follow-up of patients is lacking (Aziz et al. 1989). Furthermore, in the developing countries there has been little improvement because of failure to treat the disease owing to lack of money and medical manpower (WHO, 1982).

The success of this treatment in developed world has been due to the prevention of the emergence of drug-resistant tubercle bacilli by the use of combined therapy, in which more than one drug is given to the patient, at least in the initial months of the regimen. With this development, that is, the introduction of effective tuberculosis drugs which rapidly reverses infectiousness, the management and treatment of tuberculosis has shifted from the specialized tuberculosis sanatorium to the general hospital and out-patient clinics (Johnson and Wildrick, 1974; Goldfogel and Sewell, 1981). The responsibility for the diagnosis and monitoring of tuberculosis patients has accordingly also shifted to laboratories not specialized in mycobacteriology. Many laboratories now routinely collect and microscopically examine clinical specimens for acid-alcohol-fast bacilli (AAFB). This certainly has its shortcoming, particularly in the area of competency. In addition, if a relapse case which was once diagnosed as pulmonary tuberculosis supported by an X-ray and sputum examaination but which did not respond to the usual anti-tuberculosis chemotherapeutic regimen and if repeated excretion of bacilli in sputum was evident by smear or culture, it is generally labelled by clinicians as a 'resistant case', because acid-fast bacilli seen on direct smear were mistaken for Mycobacterium tuberculosis.

In such cases, atypical mycobacterial infection should be considered (Kotian et al. 1981). Furthermore, the primary disease manifested by Nocardia asteroides which is slightly acid-fast, is also pulmonary and varies from filterations to lobar consolidation and cavitation. This calls for another caution on direct smear and in X-ray diagnosis.

In tuberculosis the major drugs whose potency and acceptability have resulted in their widespread use are, isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA), streptomycin (SM), ethambutol (EMB) and thiacetazone (TBI) (Mitchison et al. 1988). Furthermore, this six drugs make up the World Health Organization (WHO) model list of essential antituberculosis drugs (Fox, 1988), and in addition, they are readily available in Nigeria.

In view of the current recognition of 54 species of Mycobacterium, the need for improved isolation methods to recover these from clinical specimens, the multiplicity of in vitro tests required to speciate the isolates and the requests for sensibility tests against a broad range of chemotherapeutic agents and the urgent need to diagnose tuberculosis rapidly and reliably, this work was designed to:

(1) Isolate and characterize mycobacterial species obtained from a highly selective and people of all social classes represented by patients reporting at a chest clinic of Ahmadu Bello University Teaching Hospital.
(2) Isolate and identify casual organisms other than Mycobacterium involved in pulmonary disease that mimic tuberculosis.
(3) Investigate the relationship between tuberculin skin reaction and laboratory techniques in house hold contacts of known tuberculosis patients.
(4) Investigate the sensitivity patterns of the mycobacterial isolates to the "WHO model list" of anctituberculosis drugs.

CHAPTER TWO:

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND:

The history of tuberculosis shows that the diseasee is of great antiquity and has made its impact felt throughout ages. From the various skulls and other bones which have been recovered from different parts of the world, tuberculosis was found to be evident in neolithic man (Rao, 1981). The protean nature of tuberculosis led ancient practitioners of medicine to divide phthisis (consumption) into many supposedly separate diseases referred to by many names, such as Pott's disease, lupus vulgaris, and scrofula, describing what is now known to be tuberculosis of the spine, the skin and the cervical lymph nodes respectively (Farer, et al. 1979). Rao (1981) in his contribution to the history of tuberculosis, stated that Haby Abbas of Baghdad in 980 A.D. and Cullen in 1779 recognized the infectious nature of phthisis but not until 1819 Rene Theodore Laennec, a Frenchman who was himself a consumptive and Villemin (1827 - 1892) laid the foundations of our present knowledge of the etiology of tuberculosis. They demonstrated that tuberculosis was due to a specific agent. And in 1882, Robert Koch (1843 - 1910) disclosed to the world his epoch making discovery of the tubercle bacilli.

Robert Koch was a physician in Wolsltein (now Wolstzn) a country town in Germany. After centuries of speculation as to the possible infectious nature of tuberculosis, he proved conclusively using methylene blue staining prepared in caustic potash that the cause of the disease was an infection by a specific microorganism which he later isolated. This was announced on 24th of March 1882 (Chaparas, 1982; Sakula, 1982). Later Ziehl introduced carbolic stain instead of aniline stain, while Neelsen advocated the use of sulphuric instead of nitric acid (Sakula, 1982). In this way "Ziehl-Neelsen" (ZN) stain and the "acid-alcohol-fast bacillus" AAFB were "born".

In 1890, during the Tenth International Medical Congress which was held in Berlin, Koch read a paper, "On Bacteriological Investigation" and announced that he had a substance which hindered the growth of tubercle bacilli, cured tuberculosis in infected guinea pigs and would probably be useful in the treatment of human phthisis, especially in its early stages (Sakula, 1982). This was known later as the Koch "Phenomenon" - that is, resistance of an infected animal to reinfection. This was evident in his further publication in which the substance was identified as a filtelrate from growth of tubercle bacilli on glycerol broth (Sakula, 1982; Lunn, 1983). This substance came to be known as Old Tuberculin (OT).

Old Tuberculin (OT) is the basic filterate of heat-killed bacillus and contains some impurities. It was used as a form of therapy and given subcutaneously to tuberculosis sufferers. Early results of this misleading treatment, suggested that it was a cure for the disease. However, those so treated developed several severe local and generalized infections (Lunn, 1983). In 1908, a French physician Charles Mantoux, used diluted solutions of tuberculin intradermally and, after refinements, his well-known tuberculin test was established ((Sakula, 1982; Lunn, 1983). This test, today has proved to be of enormous value as a diagnostic tool to distinguish the infected from the non-infected, especially when BCG vaccination was later introduced. This paved the way for subsequent research on allergy and immunity.

The discovery of other species of Mycobacterium soon followed. In 1898, Professor Theobald Smith of Harvard Published his findings on human and bovine tubercle bacilli and clearly demonstrated them to be different organisms (Sakula, 1982). This was soon followed by the discovery of the chicken tubercle bacillus by Coni in 1884 and was isolated by Magucci in 1890 (Cited by RaO, 1981).

Bacillus Calmette Guerin (BCG) vaccines owe their origin to the in vitro attenuation, by Calmette and Guerin, between the years 1906 and 1919, of a strain of Mycobacterium bovis (RaO, 1981; Fine 1988). The bacilli resulting from this attenuation have since been maintained by different laboratories, using many different methods, and was widely used for vaccination in Europe in the 1920s and 1930s. Doubts arose as to their safety, because of a major accident in Germany in 1929 when 72 children died of tuberculosis among 251 who had been inoculated with vaccine contaminated with virulent tubercle bacilli (Fine 1988). Doubts also arose concerning their efficacy; but they were never assessed in properly conducted trials until the mid 1930s. And despite the inconsistent results of these trials, BCG vaccines came into widespread use during the 1950s and 1960s with the support and encouragement of the World Health Organization (Fine, 1988), and it is still in use today for vaccination.

2.2. BRIEF DESCRIPTION OF TUBERCLE BACILLI:

The tubercle bacilli belong to the genus Mycobacterium (Runyon et al. 1974; Cruickshank et al. 1975). They are acid-alcohol-fast, aerobic or microaerophilic, nonspore forming, nonmotile and noncapsulated bacilli. Growth is slow requiring about four weeks on complex media at restricted temparature (Kent and Kubica 1985). The type species are M. tuberculosis and M. bovis. The individual cells of M. tuberculosis are rod shaped, 2 to 5 micrometer or more long and 0.4 to 0.6 micrometer wide (Tripathy, 1981).

The tubercle bacilli possess a thick lipoidal cell wall which has a dual effect (Chaparas, 1982). First, it renders the organism impermeable, protecting it from adverse environmental conditions such as heat, desiccation or that found within phagocytic cells or within tissues containing a drug or an antibody. Secondly, the cell wall components participate in the induction of certain activities, some of which are helpful to the host in its effort to contain progression of the disease, some of which cause tissue destruction, and some of which involve both.

The property of acid-fastness following staining with carbol fuchsin or carbol auramine is due to the presence of mycolic acid and certain unidentified "firmly bound" lipids in the cell wall (Tripathy, 1981).

Barksdale and Kim (1977) reported that acid-fastness is a property of the intact cell and that disintegration of the cell results in loss of this property. In Gram-stained preparations, tubercle bacilli appear pale violet. Their Gram positive character is independent of the mordant effect of iodine and appears to be bound up with the property of acid-fastness.

Pearson (1988) reported that Much and Khomendo in separate studies showed that ultra-fine forms of acid-fast bacilli exist which failed to show any acid-fastness in Ziehl-Neelsen's staining procedure and were 20 times smaller than the conventional rods and were spherical and filtrable. These may be found in some tuberculosis abscesses and in infected lymph nodes and have since been referred to as Much granules. The existence of these ultra-fine forms makes the diagnosis of such cases difficult and can be detected by passaging the granules through animals which cause their reversion into recognisable mycobacteria.

2.3.1 CLASSIFICATION:

Gibson, in 1953 drew attention to the acid-fast bacilli resembling mycobacteria, and showed that the orgainsms could cause disease in man, are non-pathogenic for the guinea pig and the degree of acid-fastness varies from strain to strain and with the immediate environment of the organism. The same year, Buhler and Pollack reported two cases of pulmonary infection caused by chromogenic, acid-fast organisms. Earlier, these other mycobacteria were thought to be saprophytes and Pinner (1935) had given the name "atypical mycobacteria" to these saprophytes and proved the association of certain chromogenic acid-fast bacilli with human diseases and emphasized the need for a proper bacteriological assessment (Cited by RaO, 1981). This was followed by other documentations by Xalabarder (1961), Black & Chapman (1964)

and Schaefer (1965) of several clinical diseases with X-ray findings of both pulmonary and extrapulmonary lesions attributable to these atypical mycobacteria.

Nunn & McAdam (1988) in their contribution to the pathogenicity of atypical mycobacteria stated that "Non tuberculosis mycobacteria (NTM) were only recognised as pathogens in the 1950s when they were found to be causing mostly localized pulmonary disease in middle-aged patients with pre-existing lung disease". And the first bacteriological assessment in the literature was a report of pulmonary infection caused by M. kansasii (Yue and Cohen 1966).

It is interesting to note that shortly before then, 'anonymous' mycobacteria were isolated in Lagos, Nigeria (Beer and Davis 1965). They cultured 2,852 isolates, 170 (6%) of which were diagnosed as 'anonymous' mycobacteria belonging to Runyon's Group III and IV. The geographical sources of the isolates were Lagos (1.4%), Ibadan (0.6%) Kano (1.4%) Port-Harcourt (1.5%) and Jos (1.0%).

The disease entities, including the pulmonary lesions produced by some of the atypical mycobacteria have been pathologically assessed and have been shown to closely resemble those caused by M. tuberculosis (Xalabarder, 1961; Kotian et al. 1981). They showed that the diseases produced by these organism have roentgenologic, pathologic and to some extent clinical similarities to tuberculosis, though there are distinct differences in virulence, treatment, and prognosis.

The group designated as atypical mycobacteria have been called by many other names; viz, paratubercle bacilli, pseudotubercle bacilli, unclassified, anonymous, nontuberculosis, opportunistic, tuberculoid and mycobacteria other than tubercle bacilli (MOTT) (Wolinsky, 1979). Hanks (1968) proposed the "nomer" NEAMI, for nonepidemic agents of mycobacterial infections.

The disease associated with them have been variously termed pseudotuberculosis, mycobacteriosis, non-tuberculous mycobacterial infection, and tuberculosis named after the mycobacterial specie for example tuberculosis Kansasii. However, Wolinsky (1979) is of the opinion that the most appropriate and least offensive name is nontuberculous, because it is best not to call the disease "tuberculosis due to the pronounced differences between the two infections in pathogenesis, epidemiology, prognosis and result of treatment.

Runyon (1959) labelled the atypical mycobacteria as 'anonymous mycobacteria' and classified them into four major groups. The classification was based on pigment production and speed of growth. The groups were labelled photochromogens, scotochromogens, non-photochromogens and rapid growers. This basic classification has been upheld by numerous workers (Wayne et al. 1971; Kubica et al. 1972; Meisser et al. 1974; Saito et al.1977; and Kent & Kubica, 1985). The photochromogens were defined as mycobacteria that were nonpigmented when grown in the dark but acquired a lemon-yellow colour after exposure either to daylight or artificial light (Macks, 1976; Kent & Kubica. 1985). All the species currently in this group are potentially pathogenic, and includes four species; viz, M. asiaticum, M. kansasii, M. marinum, and M. simiae (Tsukamura, 1967; Wayne & Doubek 1968; Kubica & Silcox, 1973; Kubica et al. 1973; Runyon, 1974; Macks, 1976; and Kent & Kubica, 1985).

The word scotochromogen was derived from the Greek word, skotos, meaning "in the dark"; hence the term means an organism pigmented both in the dark and light (independant of light exposure, although the latter may intensify the colour) (Wayne, 1967; Kubica et al. 1973). The species include both potential pathogens eg. M. scrofulaceum, M. szulgai, M. xenopi, and common saprophytes like M. gordonae and M. flavescens (Wolinsky, 1979; Kotian, et al. 1981; Kent & Kubica 1985; Good, 1985). M.flavescens is scotochromogenic, but it is often grouped with rapid growers, being an organism of intermediate growth rate (Wayne et al. 1971).

The nonphotochromogens are the nonpigmented species, though the name suggests that some may be pigmented but that the pigment is not affected by light (Good, 1985). There are nine species in this group (See Table I), of which the commonly encountered species include M. avium - intracellulare complex which are opportunistic pathogen for man, and M. terrae, M. gastri, and M. triviale which are saprophytes (Wayne 1967, Tsukamura, 1967, Kubica and Silcox, 1973, Kubica et al. 1973). The three groups mentioned above are all slow growers (Runyon, 1959, Wolinsky, 1979 and Good, 1985).

The rapid growers are those that yield fully matured, grossly visible and nonpigmented colonies in less than seven days of incubation (Kent & Kubica, 1985). The twenty five species in this group are shown in Table I. The species of particular interest in this group are M. chelonae and M. fortuitum which are opportunistic pathogens (Good, 1985) and M. smegmatis, M. vaccae and M. parafortuitum which are nonpathogenic to human but, with a varying frequency can be isolated alone from human materials submitted for mycobateriological examination (Kubica, 1973, Kubica et al. 1975; Saito et al. 1977; and Wolinsky, 1979).

Recently, a fifth group, that is animal pathogen has been added (Good, 1985). This group consists of six species, four of which are slow growers (Table I).

It is to be mentioned here that mycobacteria and indeed all bacteria should be precisely identified by species name but occasionally recent isolates and unknown organisms are refered to in the literature by the vernacular names such as photochromogens and scotochromogens. This according to Good (1985) is to facilitate discussion and ultimate identification of these organisms. Furthermore, species complexes e.g M. avium complex, M. terrae complex, have been used to designate two or more species whose differenciation is of no medical importance and where more precise characterisation seems to be of little clinical value (Runyon, 1974; Runyon et al. 1974; Kubica et at. 1975).

M. avium complex has been useful to group M. avium and M. intracellulare, which are difficult to differenciate with biochemical reactions, produce indistinguishable *mycobacterioses, have similar drug-susceptibility patterns,* and cross-react with delayed hypersensitivity reactions to purified protein derivatives (PPD) (Good, 1985). And in Runyon grouping (1974), M. tuberculosis complex otherwise called classical mycobacteria contains M. tuberculosis, M. bovis, and M. africanum. Tuberculosis caused by any one of these three species is indistinguishable in man, differenciation is however significant for epidemiology and public health purposes (Good, 1985). Biochemical differentiation of M. tuberculosis from M. bovis may not be difficult (Table 3), but M. africanum which consits of two groups with separate geographical distribution - one from West Africa with properties of M. bovis and the other in East Africa with properties of M. tuberculosis may present difficulty (Tripathy, 1981). This heterogeneity in biochemical properties is believed to be a bridge in the evolutionary gap between the human and bovine types (Yates et al. 1984). *Furthermore, the two medically important rapid* growers were grouped into M. fortuitum complex (Runyon, 1974), but recent studies have indicated that they can be easily differentiated in the diagnostic laboratory (Silcox et al. 1981). In addition M. terrae complex consists of M. terrae, M. nonchromogenicum and M. triviale and are essentially nonpathogenic to man, their characteristics have been studied to some extent by Wolinsky (1979) and Good (1985).

Scotochromogenic mycobacteria that appear intermediate between M. avium, M. intracellulare and M. scrofulaceum were referred to as M. avium-intracellulare - scrofulaceum intermediates "MAIS" (Hawkins, 1977). Strains in this group share similar biochemical properties (produce a deep pigment, negative for urease, Tween hydrolysis, reduce nitrate and produce a column of foam of greater than 45mm in semiquantitative catalase test).

However, Schaefer (1965 and 1967) described a seroagglutination procedure for typing strains of several species including the MAIS intermediates. Presently, three types of M avium (M. avium complex, types 1 -3), 25 types of M. intracellulare (M. avium complex, types 428), 3 types of M. scrofulaceum (M. avium complex, types 41-43) are recognised. Other workers have described the strains in the seroagglutination technique in other species of Mycobacterium (Goslee and Wolinsky, 1976; Wolinsky, 1979).

2.3.2. CURRENTLY RECOGNISED SPECIES:

In the 9th edition of Bergey's Manual of Determinative Bacteriology, there are 54 species of mycobacteria (Kent and Kubica, 1985). The species are listed in Table I, and grouped on the basis of rate of growth, chromogenicity and pathogenicity. There are nineteen obligate or opportunistic pathogens that could be isolated with a variable frequency from human sources, among which is M. laprae, which does not grow on ordinary culture media. There are six species pathogenic to animals and an additional six species among the slow growers that exist as saprophytes. Among the rapidly growing mycobacteria, there are 23 species which rarely infect humans, are commonly saprophytic, and usually are lumped together as "other rapid growers".

2.3.3. DISTRIBUTION AND SOURCES OF INFECTION

The infectious unit in tuberculosis i.e M. tuberculosis is carried on airborne droplet nuclei which are produced when persons with pulmonary tuberculosis sneeze, cough, speak or sing (Loudon and Roberts, 1968; Loudon and Spohn, 1969; Gruft et al. 1979). Primary infection by a susceptible host is usually acquired by inhalation of infected droplets, which pass down the bronchial tree into the bronchioles and alveoli beyond the mucociliary layer (ATS, 1980; Chaparas, 1982). Primary infection of the alimentary tract by M. bovis has been shown to occur from ingestion of infected milk and sometimes from the use of eating utensils or ingestion of food contaminated with either M. tuberculosis or M. bovis (Cruickshank, 1975).

Wolinsky (1979) and Gruft et al. (1980) in their review of *nontuberculous mycobacteria and associated disease*, pointed out that in contrast to tuberculosis, pulmonary disease associated with nontuberculous mycobacteria is not transmited from person, but acquired from natural sources from the environment, for example, contaminated soil and water Good (1985) also reported that human population is constantly exposed to mycobacteria through ingestion, inhaling aerosols and inoculation of traumatized skin. This was supported by the fact that mycobacteria exists and probably multiply in a wide variety of natural sources, including domestic and wild animals and birds (Singer & Rodda, 1961; Kubica et al. 1963a; Meisser & Anz, 1977). The water studies of Bailey et al. (1970); Mc Swiggar & Collins, (1974); Goslee & Wolinsk (1976); the raw milk studies of Johes et al. (1966); Matthews et al. (1976); Shehu (1988); the soil and dust investigations of Reznikor et al. (1971); Reznikor & Leggo (1974) and the animal studies of Alhaji (1976); Pritchard et al. (1977); Suwankrughasn & Leat (1977) and Anyawale, (1989) showed that mycobacteria, including the potentially pathogenic species may be found everywhere in the environment of the earth and thus may serve as a source of human diseases. Some of these environmental strains have been shown to be similar to those known to produce disease in man (Wolinsky, 1979). The isolation by Anyawale (1989) of M. avium complex in animals is significant in this regard because it is one of the two species or complexes of atypical mycobacteria which has emerged as the predominant agents of disease in man; the other being M. kansasii (Kotian et al. 1981). It is also significant to add that M. avium complex has with a high frequency been incriminated in the complication in acquired immune disease syndrome (AIDS) patients (Nunn & McAdam, 1988).

Concurrent infection with two or more species of mycobacteria has occassionally been reported in the literature (Raleigh et al. 1975; Good, 1985); and two cases of both M. bovis and Nocardia asteroides were reported by Alhaji (1976).

TABLE 1

Currently recognized Mycobacterium Species

Group	Obligate or opportunistic pathogens		Saprophytes	
Strict pathogens	M. africanum	M. leprae		
	M. bovis	M. tuberculosis		
	M. ulcerans			
Photochromogens	M. asiaticum	M. marinum		
	M. kansasii	M. simiae		
Scotochromogens	M. scrofulaceum		M. gordonae	
	M. szulgai		M. flavescens	
	M. xenopi			
Nonphotochromogens	M. avium			
	M. haemophilum		M. gastri	
	M. intracellulare		M. nonchromogenicum	
	M. malmoense		M. terrae	
	M. shimoidei		M. triviale	
Rapid growers	M. chelonae			
	M. fortuitum		M. agri	M. neoaurum
			M. aichiense	M. parafortuitu
			M. austroafricanum	M. obuense
			M. aurum	M. phlei
			M. chitae	M. pulveris
			M. chubuense	M. rhodesiae
			M. diernhoferi	M. smegmatis
			M. duvalii	M. sphagni
			M. fallax	M. thermoresis-
			M. gadium	tible
Animal pathogens			M. gilvum	M. tokaiense
			M. komossense	M. vaccae
Slow growers	M. farcinogens	M. microti		
	M. lepraemurium	M. paratuberculosis		
Rapid growers	M. porcinum	M. senegalense		

Adapted From Good (1985)

2.3.4 NOCARDIA:

Gordon (1974) described the Nocardia as aerobic, pathogenic, branched and filamentous bacteria related to the Mycobacterium. He had in 1956 (a) (b), demonstrated that some species tend to fragment into bacillary and coccoid forms, some form aerial chains of either conidia or arthrospores, Gram positive and that some species are weakly or partially acid-fast.

Nocardia grows well but slowly on all common laboratory media including Lowenstein-Jensen medium, and is usually not killed by the digestion procedure used on sputa for culture of tubercle bacilli (Gordon, 1974; Freeman, 1983). Growth rates are comparable to those of the rapidly growing mycobacteria and the optimum temperature for growth is 30-37°C. The species are non-encapsulated and differenciation is by their biochemical action upon casein, tyrosine, xanthine and starch agar plates (Gordon & Mildred, 1954); urea agar slants and tubes of gelatin and bromocresol purple (BCP) milk and by whether they produce acid from certain sugars (Georg et al. 1961).

They are generally opportunistic pathogens causing severe, suppurative pulmonary infection, often simulating tuberculosis (particularly N. asteroides) cutaneous and subcutaneous abscesses (N. asteroides, & N. brasiliensis);secondary blood stream invasion often fatal with involvement of the meninges and brain, kindney and other organs (N. asteroides) (Cyamon & Palmer, 1981; Freeman, 1983). Pulmonary or generalized nocardiosis has become increasingly prevalent, especially in patients with severe underlying diseases, and who are receiving intensive medical theraphy, particularly implicated are steroids and antineoplastic drugs (Young et al. 1971).

The organism could be found in clinical specimens, lesions of animals eg bovine mastitis and soil. Transmission is by direct inhalation of contaminated dust or by direct traumatic contact with contaminated soil or vegetation (Alhaji, 1976).

In a study conducted by Alhaji (1976), eleven N. asteroides were isolated from 81 tissue specimens collected from cattle in different areas of the northern part of Nigeria.

2.4 DIAGNOSIS OF TUBERCULOSIS:

Tuberculosis has for a long time been recognized as an infectious disease that usually presents with characteristic symptoms (Wolinsky, 1979; ATS, 1981). However, many patients including some with extensive disease, have non-specific symptoms. (Dongrey et al. 1981; Chandrasekhar et al. 1981). The asymptomatic patients and the person who does not recognize symptoms can be identified only through a history of exposure, an abnormal chest roentgenogram, a significant reaction to tuberculin skin test, demonstration of acid-fast bacilli in samples by direct microscopy and cultures (Cruickshank et al. 1975; ATS, 1981).

Any organ of the body (Siddiqi et al. 1976; ATS, 1981) including the bone and joint (Davidson et al. 1970), meninges, peritoneum, pericardium, larynx and skin can be involved in tuberculosis. Thus organs involvement may not present with typical signs and symptoms, and even symptoms of pulmonary tuberculosis do not form a clear-cut syndrome; they are common to many non-tuberculous conditions (Dongrey and Bhatia, 1981). In view of this a definitive diagnosis of tuberculosis requires the demonstration of acid-fast bacilli in an acid-fast staining, cultural growth, isolation and identification of the Mycobacterium (Kent and Kubica 1985).

The technical procedures for the diagnosis of tuberculosis by clinical features and radiology have been documented by Achar (1981). However, for the purpose of this review tuberculin skin test and demonstration of tubercle bacilli which essentially involves the mycobacteriology laboratory will be discussed.

2.4.1 TUBERCULIN SKIN TEST:

Mycobacterial infection produces delayed hypersensitivity to certain products of the organism contained in culture extracts called "tuberculins" (ATS, 1981; Achar and Viswanathan, 1981). This cell-mediated reaction is manifested by induration due largely to cellular infilteration at the site of the antigen injection in sensitized persons (Mackaness, 1968). Such persons are termed "reactors". Not all reactors are infected with M. tuberculosis or M. bovis. Infection with mycobacteria other than the classical mycobacteria may cause cross-reactions to tuberculin prepared from M. tuberculosis (Ten Dam & Hitze, 1980).

Various purified protein derivatives have been prepared from Mycobacterium other than M. tuberculosis and these are variously known as mycobacterins, sensitins, or elicitins (Edwards et al. 1965; Ten Dam & Hitze, 1980; Pahano et al. 1982; Pust & Erickson, 1989). The antigens that have been identified and in current use have the following designations: PPD -S, which is the international standard tuberculin derived from M. tuberculosis (Pust and Erickson, 1989); this has been replaced by Tween-stabilized tuberculins which is known as PPD-RT-23 in most of the world, or PPD-T in North America; PPD-G, PPD-F and PPD-Y are derived from M. scroflaceum, M. fortuitum and M. kansasii respectively (Ogunmekan, 1980; Pahano et al. 1982); PPD-A, PPD-PL from M. avium and M. marimum respectively (Ogunmekan, 1980); and PPD-B from M. intracellulare (Pahano et al. 1982).

Wolinsky (1979) in his review, and in separate studies by Edwards et al. (1965) and Magnusson (1961) using purified protein derivatives prepared from various acid-fast bacilli with guinea pigs as their model animals showed that mycobacterial sensitins possess a certain type of species specificity and that humans or animals infected with a given Mycobacterium usually show stronger reactions to sensitins produced from homologous Mycobacterium than to sensitins produced from other species.

Tuberculin skin test is a diagnostic aid in the evaluation of the individual patient (detecting reactors) and most useful in determining the prevalence of tuberculosis infection in populations (Holden et al. 1971) Patients with a positive reaction should be presumed to be at risk of having active disease even though there are no signs and symptoms (ATS, 1981). The test is also valuable when it is repeated periodically in the surveillance of tuberculin-negative persons likely to be exposed to tuberculosis (Thompson et al. 1979).

A converter is defined by American Thoracic Society (ATS, 1981) as a person whose tuberculin reaction has increased by at least 6mm, from less than 10mm to 10mm or more in diameter, within 24 months (period of greatest risk of the newly infected person to develop disease). It is noteworthy that the absence of a reaction to tuberculin skin test does not exclude a diagnosis of tuberculosis (Kent & Schwartz, 1967; Howard et al; 1970; Holden et al. 1971; Comstock & Woolpert, 1978); and therefore a definitive diagnosis ultimately requires the demonstration of Mycobacterium from the appropriate specimen.

2.4.1.1 ADMINISTRATION AND READING:

The methods of performing the tuberculin skin test according to Achar and Viswanathan (1981) and Lunn (1983) include (i) Heaf multiple puncture test (ii) Mantoux intracutaneous test and (iii) Vollmer patch test.

The recommended antigen is purified protein derivative (PPD-T) tuberculin, stabilized with Tween 80 and standardized by biological assay to 5 tuberculin units (5TU) containing 0.1 mg of PPD in 0.1ml saline (Richards et al. 1979; Ogunmekan, 1980; Lunn, 1983). And the international standard technique is the Mantoux (Pust & Erickson, 1989). The technique is well documented by Atwell et al. 1961; Edwards et al. 1965 and Lunn, 1983); and the time and technique of reading postinoculation, the size of the induration have been described by Wijsmuller (1966) and ATS (1981).

2.4.1.2 INTERPRETATION AND FACTORS AFFECTING SKIN TEST REACTIONS:

The significance of a given amount of induration should be based on data obtained from a large epidemiologic surveys of patients with documented tuberculosis, mycobacterial disease caused by other organism, and individuals without known tuberculous disease (Rust & Thomas, 1975; Ten Dam & Hitze, 1980). For example in the United States, the available data indicate that a reaction of 10mm or more is significant when screening populations for infection (ATS, 1981). However, when the survey is intended to select those individuals suspected to have tuberculosis or close contacts of individuals with tuberculosis, a reaction of 5mm or more is considered significant. Because of this variation in the interpretation of results, ATS (1981) states "the significance of a reaction (tuberculosis skin test) is determined not by its size but by the clinical circumstances and the intended use of the result.

Holden et al. (1971), Schaefer (1972) and Lunn (1983) outlined reasons for "anergic" state. False positive tuberculin skin test could also occur, causes of which have been outlined by Bernard & Duboczy (1961) and Ten Dam & Hitze (1980).

Levels of tuberculin sensitivity in individuals before and after Bacille-Calmette-Guerin (BCG) is another factor that needs mentioning. In a mass BCG vaccination programme, post-vaccination tuberculin sensitivity could be used to evaluate the success or otherwise of the programme because the "Conversion" of tuberculin reaction from negative to positive after vaccination signals the acquisition of immunity (WHO 1959; Guld, 1966; Wijsmuller, 1966; Tem Dam & Hitze, 1980). The tuberculin test reaction after BCG vaccination however, cannot be distinguished from that due to infection by atypical mycobacteria (Guld et al. 1968; ATS, 1981). It is therefore suggested that a history of vaccination is obtained from patients so as not to alter the guidelines for the interpretation of the reaction.

The booster phenomenon, that is, the enhanced tuberculin respons after repeated testing, has long been recognized in those infected with M. tuberculosis or individuals who have been vaccinated with BCG (Ferebee & Mount, 1963; Comstock & Woolpert, 1978; Valenti et al. 1982). This phenomenon has also been appreciated in those with cross-reacting sensitivity to non-tuberculous mycobacteria (Knight et al. 1963; Raj Narain et al. 1968; Glassroth & Thompson, 1977; Richards et al. 1979; Thompson et al. 1979); while repeated testing of uninfected persons does not sensitize them to tuberculin (ATS, 1981). Thompson et al. (1979) reported that the booster effect can be seen on a second test done as soon as a week after the initial stimulating test and can persist for a year and perhaps longer in infected individuals. On the basis of this, they recommended that repeated tuberculin testing should be incorporated into a hospital control programme to permit screening of new infections from boosted reactions. Persons who do not boost when given repeat tests at one week, but whose tuberculin reactions change to positive after one year, should be considered to have newly acquired tuberculous infection and managed accordingly.

2.4.2 DEMONSTRATION OF TUBERCLE BACILLI:

The demonstration of tubercle bacilli in clinical specimens is essential for the disease, and since the time of Koch, microscopy has been employed for this purpose (Lincolyn & Gilbert, 1972). Kent & Kubica (1985) observed that it is perhaps the easiest and most rapid procedure that can be performed in the laboratory to detect the presence of acid-fast bacilli. However, it is less sensitive than culture (Yeager et al. 1967; Bannister et al. 1976; Burdash et al. 1976), and except of recent, acid-fast bacilli seen in direct smears were often mistaken for M. tuberculosis (Lincolyn & Gilbert, 1972). In spite of these shortcomings microscopy can be helpful in the following ways in the mycobacteriology laboratory: (a) as a diagnostic indicator especially when associated with significant clinical manifestation (Mitchson, 1972; Bannister et al. 1976). (b) rapid identification of

infectious cases, that is, those that are smear positive (Greenbaum et al. 1980). (c) to follow the progress of tuberculosis patient on chemotherapy (Gunnels et al. 1974). (d) it is of vital importance as regards to the patient's discharge from the hospital, or return to gainful employment (Cashman et al. 1970). (e) to confirm that cultures growing on media are acid-fast (Kent & Kubica, 1985) and; (f) in determining the appropriate dilutions of sediments for direct drug susceptibility tests (Canetti et al. 1969; Kent & Kubica, 1985).

2.4.2.1 SMEAR PREPARATION AND STAINING METHODS:

Chandrasekhar & Padmanabla (1981) enumerated the important points in the preparation of smears, some of which include the use of new, clean and unscratched slides, selection of purulent or necrotic material and the preparation of a thin evenly spread smear, and fixing after air-drying by passing the smear over a bunsen flame. The current practice is to use the deposit obtained after the appropriate concentration and decontamination techniques (Kent & Kubica, 1985).

Commonly used decontaminants are the modified Zephiran trisodium phosphate technique (Krasnow & Kidd, 1965); cetylpyridinium chlaride and sodium chloride method (Smithwick et al. 1975); modified sodium hydroxide method of Petroff (Cruickshank et al. 1975); sodium hypochlorite or the household chloride bleach (Smithwick & Stratigos, 1978); polycarbonate membrane filter (Smithwick & Stratigos, 1981); glutaraldehyde technique (Goldfogel and Sewell, 1981).

2.4.2.2 CULTURAL PROCEDURE:

Numerous mycobacteriological culture media are available most of which fall into two general categories of egg-potato-base or serum (albumin) and agar-base media (Kubica and Dye, 1967; Kent & Kubica, 1985). The most popular egg-based media are the buffered egg-potato medium of Lowenstein-Jensen and egg-potato flour medium of the American Trudeau Society; and among the agar-based media, Middlebrook 7H10, Middlebrook

7H11 and Dubos oleic-albumin agar are in common use (ATS, 1981; Kent & Kubica, 1985). Kent & Kubica (1985) recommended that both an agar-base and an egg-base media be used for primary isolation of mycobacteria so as to increase the efficiency of isolation.

An atmosphere of 5-10% carbondioxide has been recommended for the incubation of inoculated media (Whitcomb et al. 1962). Their findings showed that it initiated growth in various Middlebrook agars and stimulated earlier luxuriant growth on egg-base media.

2.4.2.3 IDENTIFICATION OF MYCOBACTERIUM:

A multiplicity of mycobacterial species, both saprophytes and potential pathogens, may be isolated from humans and the cooperative studies of clinicians and bacteriologist over the past decade, have made it possible to designate the potential clinical significance of most species of mycobacteria as shown in Table 2 (Wolinsky, 1979; ATS, 1981).

The differential characteristics that are useful in the identification of these species are listed in Table 3. The techniques and interpretation of these tests have been developed and described in several publications (Kubica & Dye, 1967; Wayne & Doubek 1968; Kubica, 1973; Kent & Kubica, 1985). Basically, all the schemes of identification begin with the observation of pigmentation and speed of growth to make the primary decision on the selection of further tests. ATS (1981) and Good (1985) have suggested that in a situation whereby an isolate does not match any of the pattern in Table 3, the tests should be repeated and with a second failure the isolate should be referred to a reference laboratory for definitive identification.

The major task of any mycobacteriology laboratory is to identify M. tuberculosis, the main causative agent of tuberculosis. The biochemical characteristics of this organism have been described by Warren et al. (1983) and Kent & Kubica (1985).

TABLE 2
MYCOBACTERIA RECOVERED FROM VERTEBRATES INCLUDING HUMANS

	Potentially Clinically Significant		Usually Not Significant	
Group	Species	Synonyms (remarks)	Species	Synonyms
	M. tuberculosis * M. bovis* M. africanum* + M. microti		M. bovis BCG*	
Photo-Chromogen	M. kansasii* M. marinum* M. simiae* M. asiaticum*	(Yellow bacillus)M. luciflavum M. balnei, M. platypoecilus M. habana		
Scoto-Chromogen	M. scrofulaceum* M. szulgai*	M . marianum, M. paraffinicum	M. gordonae* M. flavescens*	M. aquae, tap water scotochrome
Nonphoto-Chromogen	M. avium* M. intracellulare* M. ulcerans* M. xenopi* M. malmoense* M. haemophilum*	(Battey bacillus)M. brunense M. buruli M. littorale (Needs hemin for growth)	M. gastri* M. nonchromogenicum* M. terrae* M. triviale*	
Rapid Grower	M. fortuitum* M. chelonei* M. chelonei subsp. abscessus*	M . ranae, M. giae, M. minetti M. borstelense M. runyonii, M. abscessus	M. phlei* M. smegmatis* M. vaccae*	M. moelleri
Others	M. paratuberculosis M. leprae* M. lepraemurium*	M. johnei (needs mycobactin for growth) Hansen's bacillus (no growth on lab media) Stefansky's bacillus (no growth on lab media)		

* Indicates species found in humans
\+ An organism intermediate between M. tuberculosis and M. bovis: status as distinct species being evaluated.

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It is required that strains of M. tuberculosis be screened for catalase activity as this may provide the clinician with valuable information related to isoniazid resistance even before drug susceptibility tests are performed, since isoniazid resistant stains may lose catalase activity when heated at 68°C for 20 minutes.

2.4.3 RAPID METHODS FOR THE IDENTIFICATION OF MYCOBACTERIUM:

Satisfactory specificity and sensitivity have been achieved using microscopy in the diagnosis of pulmonary tuberculosis particularly in smear-positive cases (Greenbaum et al. 1980). However, it may take several weeks before a positive sputum culture is obtained, moreso in smear-negative disease and in extrapulmonary tuberculosis, particularly in children where haematogenous spread may cause meningitis and osteomylitis (Ivanyi et al. 1988).

A significant number of patients who may be infected or may become sources of infections evade detection because of this difficulty, moreso in poorer countries where bacteriological culture is not routinely done . In view of this, various attempts are being made in search for rapid methods for the identification of mycobacterial species. In addition to tests like niacin, catalase and nitrate reductase that can be performed and read the same day, numerous workers have shown that the detection of B-glucosidase (David & Jahan, 1977) urease, penicillinase, trehalase and cephalosporinase (David et al. 1981; Frebault et al. 1982) were of value in distinguishing species of mycobacteria. Other assays for enzymatic activity by Grange (1989) also suggest a promising procedure for the rapid identification of mycobacteria.

Laszlo & Siddiqi (1984) have developed techniques for both rapid detection and differenciation of tubercle bacilli from opportunistic and saprophytic species by selective susceptibility to 5 microgram per millilitre p-nitro-acetylamino-B-hydroxypropiophenone (NAP) in Middlebrook 7H12 medium.

The identities of opportunistic pathogens and saprophytes were suggested by growth patterns and cell morphology. Absolute identification of species could not be made by this method. Tisdall et al. (1979 and 1982) & Guerrant et al. (1981) reported that the analysis of fatty acids by gas-liquid chromatography (GLC) is a reliable procedure for the identification of mycobacteria in the clinical laboratory. They isolated 335 strains from clinical samples and employed GLC for their analysis. A total of 320 were correctly identified without further testing. Geuerrant et al. (1981) analyzed fatty acid mythyl esters obtained by acid methanolysis and hexane extraction and reported that GLC is effective for species identification. Specific application of thin-layer chromatography (TLC) to the identification of mycobacteria has been recommended by Jenkins (1981). It may offer a promising tool for the serotyping of mycobacteria for epidemiological studies.

Serological studies with tuberculin, employing crude or partially purified mycobacterial extracts have been explored by numerous workers in a variety of immunological techniques; and have promising solution to the knotty problem of rapid diagnosis of tuberculosis patients. Reggiardo and Vazquez (1981) investigated the possibe use of antigens prepared from M. tuberculosis in the serological diagnosis of mycobacteriosis. They employed enzyme-linked immunosorbent assay (ELISA) and passive haemagglutination techniques, using three purified serologically active glycolipid antigens (SAG) from M. tuberculosis BCG. The two techniques appear to be sensitive and specific (96.6% specificity and 86.4% sensitivity for ELISA and specificity of 96% and sensitivity of 86.6% for haemagglutination) in the detection of mycobacterial diseases in patients and in healthy individuals. In a similar study by Benjamin et al. (1984) five mycobacterial antigens were compared in an ELISA for serodiagnosis of tuberculosis. The antigens were an unheated sterile culture filterates of M. tuberculosis, tuberculin PPD from M. tuberculosis (PPDa), purified cytoplasmic protein antigen 5 and 6 from M. tuberculosis and a PPD prepared from M. kansasii (PPDk).

Analysis of the titre obtained from each of the antigens were significantly different in tuberculosis and in control groups, and the most accurate test was obtained with anigen 5. M. tuberculosis PPD, M. tuberculosis antigen 6 and M. tuberculosis culture flterate were, in descending order, less accurate. They recommended the use of antigen 5 in ELISA for the serodiagnosis of tuberculosis in populations suspected of having tuberculosis. In an earlier study, stroebel et al. (1982) and kalish et al. (1983), suggested the use of antigen 5 for the serodiagnosis of tuberculosis using ELISA. The suggestion was upheld by Balestrino et al. (1984), who measured lgG antibody to M. tuberculosis antigen 5 and tuberculin PPD by ELISA in active pulmonary tuberculosis and non-tuberculous subjects, and found that the techniques were reliable.

The characteristics of monoclonal antibodies (MABs) abc to distinguish tubercle bacilli and other mycobacteria have been reviewed by young (1988). Affinity chromatography using MAB directed against plasma membrane antigens of M. tuberculosis has provided reagents for immunological tests with limited diagnostic success (Daniel & Anderson, 1978).

The diagnostic potential of serological tests in tuberculosis has been bedevilled by cross-reactivity of antigens within the mycobacteria and other bacterial genera (Hattikudur & Kamat, 1985; young, 1988). In addition, sera from healthy infants, whose subsequent tuberculin test proved negative, have been shown to contain antibodies which bind to soluble extracts of M. tuberculosis (Ivanyi et al. 1988). These problems along with the cumbersome procedure in the preparation of mycobacterial antigens have prevented the serodiagnosis of tuberculosis (Reggiardo & Middlebrook 1974(a)&(b)). It appears however, that with the present progress, microscopic and bacteriological analysis of clinical samples will soon be substituted by more rapid techniques in the diagnosis of tuberculosis.

2.5 ANTI-TUBERCULOSIS CHEMOTHERAPY AND DRUG SENSITIVITY TESTS:

One of the major medical advances of the twentieth century has been the development of anti-tuberculosis drugs, and when given in optimal combination, will cure almost every patient suffering from tuberculosis (Citron, 1982). The effectiveness of chemotherapy in tuberculosis however, is dependent upon many factors, among which are; the tubercle bacilli and their metabolic activity (Dickinson & Mitchison, 1981), the size of the bacillary population in the lesion (Mitchison, 1984), the pharmacodynamics of the drugs, the site of the disease and the compliance of the patient to instructions (Gangadharam, 1981; Kent & Kubica, 1985). These also explain the treatment failure and relapse encountered in anti-tuberculosis chemotherapy (Mitchison et al. 1988).

In addition to the prevention of both treatment failure and relapse, anti-tuberculosis chemotherapy is also aimed at complete sterilization of the lesion in the shortest time possible (Dickinson & Mitchison, 1981). This is also the rationale for short-course chemotherapy (SCC) with multiple drug combinations (Jindani et al. 1980; BTA,1982). An essential element required for the achievement of this goal is to administer effective treatment regimens (ATS, 1981). This is best achieved by performing drug sensitivity test, particularly in communities where primary drug resistance is more than five percent (ATS,1981), or in instances of relapse or retreatment cases, or when consideration is being given to change in the drug regimen because the patients specimens remain positive after four to five months of treatment or show a sharp and persistent increase in acid-fast bacilli after an initial decrease or when primary drug resistance is suspected (Mitchison, 1984).

2.5.1 BRIEF DESCRIPTION OF SOME ANTI-TUBERCULOSIS DRUGS AND THEIR MODES OF ACTION:

(a) ISONICOTINIC ACID HYDRAZIDE (ISONIAZID) (INH)

It is bactericidal against actively growing tubercle bacilli, and less effective against resting tubercle bacilli (Jindani et al. 1980). The results of several studies suggest that the primary drug effect is to inhibit synthesis of mycolic acid, an important component of the cellwalls of mycobacteria (Winder and Collins, 1970).



FIG 1: Isonicotinic Acid Hydrazide (INH) (Lorian, 1966).

Toida (1969) showed that a strain of avian mycobacteria resistant to INH produces an enzyme hydrazidase which hydrolyses the INH to hydrazine and isonicotinic acid. Resistance to this drug, as with streptomycin, emerges rapidly and there is no cross resistance with other antibiotics or chemotherapeutic agents except the compounds of hydrazide (Mitchison, 1984). It may interfere with metabolic events requiring pyridoxal cofactor eg. transaminases and therefore administered concomitantly with pyridoxine (Hoeprich, 1976).

(b) **STREPTOMYCIN:** This was the first drug shown to be effective against tuberculosis in humans and like any other aminoglycosides, streptomycin inhibits the synthesis of protein (Pratt, 1977). It is bactericidal and less so than INH (Jindani et al. 1980).

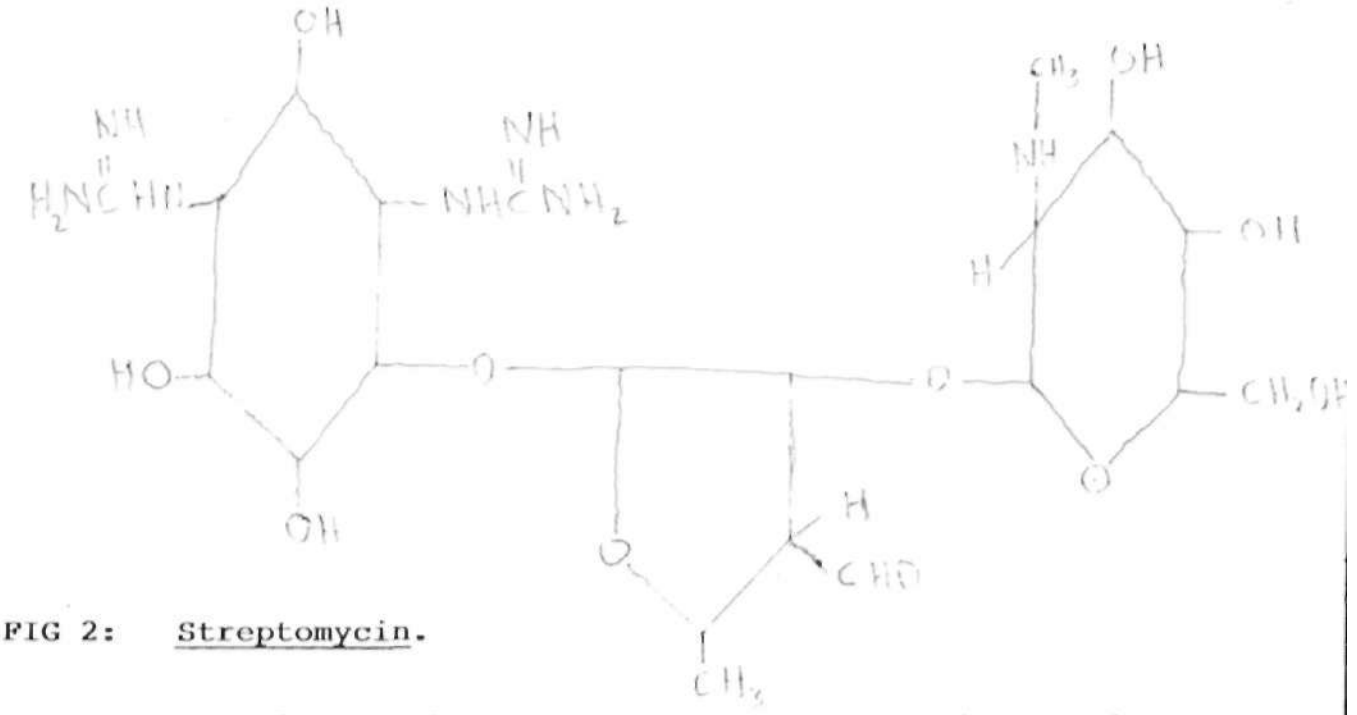


FIG 2: Streptomycin.

The toxic effects of streptomycin on the kidney and ear (balance and hearing) and the necessity for parenteral administration have made it less attractive than oral drugs (Pratt, 1977). In addition, its use in the initial therapy of tuberculosis is declining as the effectiveness of regimens without the drug has been established (Fox & Mitchison, 1975).

(c) **ETHAMBUTOL:** It is a synthetic antituberculosis drug and has the following chemical structure (Pratt, 1977):

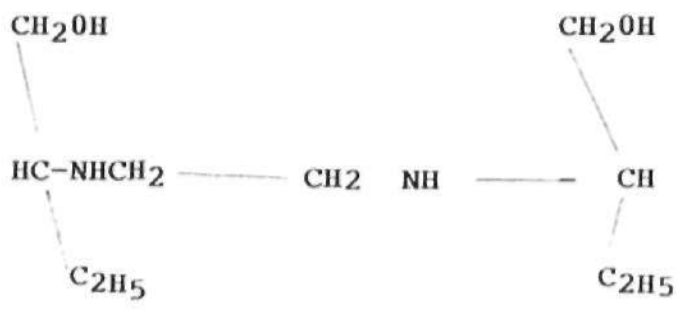


FIG 3: Dextrorotatory-2, 2'- (Ethylenediimino)-di-I-butol.

It is a secondary line anti-tuberculosis drug and is used in association with INH or streptomycin to prevent resistance, and shows some activity against some atypical strains (Pratt, 1977).

(d) **RIFAMPIN:** This is one of a large number of semisynthetic derivatives of the antibiotic rifamycins (Mitchison et al. 1988). It is very active against mycobacteria, including some atypical strains, Gram positive organisms and Neisseria (Pratt, 1977). It acts by inhibiting RNA synthesis (Wehrli and Staehelin 1971).

The group which characterised rifampin is:

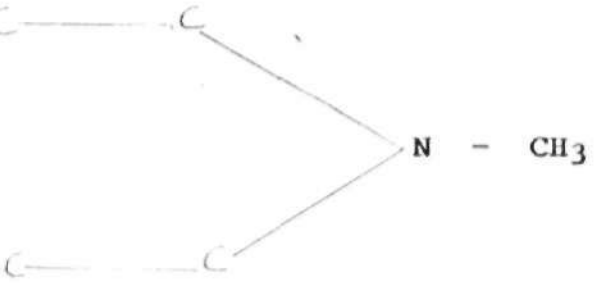


FIG 4: Rifampin

It is attached at the 3 position to the common portion of the molecule shown below, and seven other rifamycins that are in current use have ben detailed by Mitchisonn et al. (1988).

Kashim Ibrahim Library,
Ahmadu Bello University,
Zaria - Nigeria

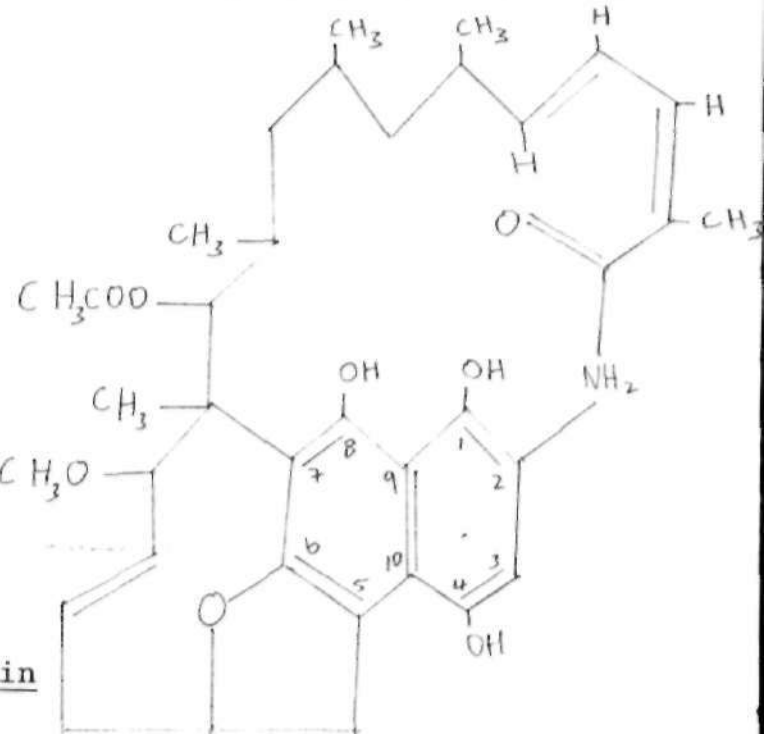


FIG 5: A Molecule of rifamycin

(e) **PYRAZINAMIDE:** This is a hydrazide of pyrazinic acid, mycobacteriostatic in effect, less active then INH, and resistance emerges rapidly, but there is no cross-resistance with INH (Lorian, 1966).

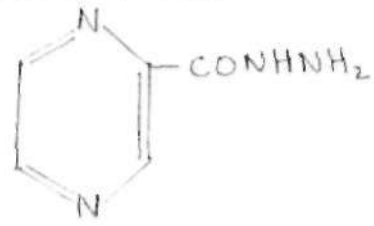


FIG 6: Pyrazinamide

2.5.2 TECHNIQUES OF MYCOBACTERIA DRUG SENSITIVITY TESTS

There are three indirect methods for determining drug sensitivity (Canetti et al. 1969; Kent & Kubica, 1985), viz the absolute concentration method, the resistance ratio method, and the proportion method. The three methods have been described in separate reviews by Canetti et al. (1963 and 1969), Kubica et al. (1975) and Kent & Kubica (1985).

The submerged disc technique which is based on the proportion method of sensitivity testing was first described by Wayne & krasnow (1966). Its use has been critically evaluated by Griffith et al. (1967 & 1971); and Hawkins (1976). It has been shown to obviate many of th problems associated with sensitivity testing, such as instability of the drugs and imprecision in weighing and diluting the drugs. Paper discs containing appropriate concentrations of the primary drugs are currently available commercially (Kubica et al. 1975; Kent & Kubica, 1985).

Various media, such as Lowenstein-Jensen medium (Kubica et al. 1975; Gangadharam, 1981), 7H11 medium (Kent & Kubica, 1985) and Dubos oleic acid agar (Haas et al. 1973) have been used in performing drug sensitivity tests. However the medium of choice is 7H10 agar, because of its simple composition, ease of preparation and its transparency which enables the earlier detection of and more precise quantitation of colonies (Kent and Kubica, 1985). This is in direct contradiction

to the recommendations of the WHO Expert Committee that recommended the use of Lowenstein-Jensen medium without potato starch (Canetti et al. 1963).

2.5.3 RAPID SENSITIVITY METHODS

In view of the fact that sensitivity test results take a long time to be available, several attempts have been made to evolve rapid testing procedures for drug sensitivity. One of such methods is the direct method (Devaki et al. 1972; Kent & Kubica, 1985). In contrast to the indirect methods that were formerly described, the inoculum for the direct method is a digested and decontaminated clinical specimen in which acid-fast bacilli had been demonstrated in stained smears. The inoculum size is adjusted on the basis of the numbers of bacilli observed in the smear, and inoculated into the drug-free and drug-containing slopes (Kent & Kubica, 1985). It provides an earlier test result because it does not require a subculture of the organism on a primary isolation medium as in the indirect method.

Several rapid radiometric methods for the sensitivity testing of mycobacteria have been reported (Middlebrook et al. 1977; kertcher et al. 1978; Siddiqi et al. 1981; Snider et al. 1981). Middlebrook and associates (1977) used a medium containing radiolabeled palmitate as a carbon source. Early detection of growth of M. tuberculosis was by measuring the conversion of the substrate to $^{14}CO_2$ in an ionized chamber. This technique has been the most widely accepted radiometric methods, as the procedure was compared to the standard growth sensitivity tests, and the results appeared to be in agreement.

2.5.4 DRUG RESISTANCE:

This was defined by Mitchison (1962) "as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with

the drug" It can emerge to all of the antibacterial drugs used in the treatment of mycobacterial disease. It occurs most commonly when a drug is given alone and when the viable bacterial population in the lesion is large (Mitchison, 1984). Other reasons for the development of drug resistance have been highlighted by Gangadharam (1981) and Parsons (1989). In any case, it arises in M. tuberculosis exclusively by spontaneous mutation that occur at random in the bacterial population and not by adaptation after exposure to the drug (David, 1970; Gangadharam, 1981). A certain proportion of drug-resistant mutants therefore exists in all populaltions of drug-sensitive tubercle bacilli (Canetti et al. 1969); Kent & Kubica, 1985). When single drug therapy is used in the treatment of tuberculosis, the large population of sensitive organism is inhibited or killed, thus leaving those few spontaneously occuring mutants (unaffected by the drug) to multiply until they comprise the majority of the bacterial population (Canetti et al. 1969; Mitchison, 1984; Kent & Kubica, 1985).

Mutation to drug resistance is thought to occur in bacterial chromosomes (Mitchison, 1984). To corroborate this, Mitchison et al. (1989) showed that patterns of drug resistance in mycobacteria are not random but occur through linked mechanism. The isolation of plasmids (Mizuguchi et al. 1981; Meissner & Falkinham, 1986) and bacteriophage (Timme et al. 1984) have been described (though not from strict pathogens: M. tuberculosis, M. bovis, M. africanum). This lends support to the suspicion that mechanism of drug resistance to antimycobacterial drugs in tubercle bacilli could be chromosomal.

2.5.5 NOMENCLATURE OF DRUG RESISTANCE:

Epidemiologically, isolation of resistant organisms from patients is classified into three in relation to the history of previous therapy (Canetti et al. 1969; Gangadharam, 1981; Mitchison, 1984). These are (i) primary drug resistance, that is, when the strains of mycobacteria are either naturally

resistant to the drug, or have acquired resistance in another person as a result of ineffective chemotherapy. (ii) acquired drug resistance, that is, when the patients had had drug-sensitive bacilli to start with; but later become drug-resistant due to inadequate or inappropriate chemotherapy, or irregular drug-taking (iii) initial drug resistance, denotes drug resistance in patients who give no history of previous chemotherapy, but in reality consists of true primary resistance, as well as acquired resistance in patients who have either concealed a history of previous chemotherapy or have received chemotherapy for example isoniazid for a cough, without being aware of the medication. Initial drug resistance is gaining great practical importance in recent times, because it takes elaborate efforts to check conclusively the possibility of previous but unreported treatment (Gangadharam, 1981).

The importance of the three types of drug resistance in theory and in practice, their impact on the chemotherapy and epidemiology of tuberculosis have brought forth a vast literature which have been reviewed by numerous workers (Hobby, 1962 & 1963; Canetti, et al. 1969; Gangadharam, 1981).

CHAPTER THREE:

MATERIALS AND METHODS:

3. MATERIALS AND METHODS:

3.1. ISOLATION AND CHARACTERISATION OF MYCOBACTERIA IN TUBERCULOSIS PATIENTS

3.1.1. **CLINIC AND PATIENTS:** The study was carried out in Ahmadu Bello University Teaching Hospital (ABUTH) Zaria in a tuberculosis chest annexe attached to the hospital. The annexe is a 40 bed hospital, consisting of eight blocks; four for males and the other four for females. It is a chest reference centre and it receives patients mostly from the northern states of the country. The clinic runs on Thursdays under the supervision of a senior consultant physician assisted by two medical officers, some nurses and other paramedical staff.

The patients were those already diagnosed tentatively either by microscopic examination of the sputum or by chest X-ray lesions. They were therefore referred to the chest clinic for treatment and management. Those with extrapulmonary tuberculosis were from ABUTH, mainly from the renal clinic and the medical wards.

3.1.2. **SPECIMEN COLLECTION:** Wide-mouth, screw-capped sterile glass containers were used for specimen collection and storage. The bottles were given each to the patients and they were instructed to spit their expectorants into them, after rinsing their mouths with clean water to minimize residual food particles and other materials that might contaminate the specimen or inhibit growth of any acid-fast bacilli that might be present in the samples. About 10ml. or more of the exudative material were collected from each patient. In suspected cases of renal tuberculosis, whole voided early morning urine samples were collected in sterile bottles large enough to contain voided urine at a time. Two or three of such samples were collected

per patient where possible. The urine samples from each patient were pooled and treated as a single specimen. In other forms of extrapulmonary tuberculosis body fluids, such as pus, pleural and peritoneal fluids were collected by a medical officer. About 20ml of peritoneal or pleural fluid was aseptically aspirated into a sterile 50ml. centrifuge tube. 2ml. of one per cent sodium citrate was added to bloody or serous effusions to prevent clotting.

Three hundred samples consisting of 229 sputa, 26 urine, 6 peritoneal fluids, 32 pleural fluids, 3 pus, 4 lymph nodes and 1 bone marrow were collected during the course of this study. Four of the peritoneal fluids, 30 pleural fluids and one lymph node were collected from patients suspected of miliary tuberculosis and admitted in the medical wards of the hospital between September, 1987 to December, 1989. All the samples were properly numbered and labelled with the name of the patient, hospital number, sex, age, date, time of collection, type of specimen and accompanied with a request form. They were transported to the Mycobacteriology laboratory which is about seventeen kilometres away from the clinic soon after collection. Where it was not possible to bring them to the laboratory immediately after collection, the specimens were kept in a refrigerator located in the chest annexe. Processing was commenced immediately on geting to the mycobacteriology laboratory.

3.1.3. OTHER SOURCES OF MYCOBACTERIAL SPECIES:

Eighteen mycobacterial isolates obtained from a previous study in the Faculty of Veterinary Medicine (FVM) were also included in the study presented here. Twelve of the isolates were obtained from sour milk (nono) and the rest from sputa of cattle rearers.

3.1.4. DECONTAMINATION, DIGESTION & CONCENTRATION TECHNIQUES:

Decontamination was carried out using Petroff's modified method (Cruickshank et al. 1975) of using four per cent sodium hydroxide. Digestion of tenacious samples was carried out by adding 0.25g N-acetyl-L-cysteine (NALC) which is a mucolytic agent to 50.0ml of the four per cent sodium hydroxide (Kubica et al. 1963[b]; Kent & Kubica, 1985). An equal volume of the digestant-decontaminant mixture was added to the samples and then mixed thoroughly until digestion was completed. The specimen containers were then placed in the incubator at 37°C for 30 minutes and shaken every five minutes over the period. At the end of the 30 minutes, the samples were separately transfered aseptically to a screw-capped 50ml. Centrifuge tubes and filled with sterile distilled water to minimize the tuberculocidal action of sodium hydroxide, to lower the specific gravity of the digested material and to further neutralize any toxic substance that might be present in the sputum.

The samples were then centrifuged at 3,000 revolutions per minute, for 20 minutes, in order to concentrate the mycobacteria (Kent & Kubica, 1985). The supernatant was poured off aseptically into a bottle containing izal disinfectant. The sediment was then neutralized by adding a few drops of 8 percent hydrochloric acid until it was neutral. The neurality was confirmed using one per cent phenol red as indicator (Cruickshank et al. 1975). The resuspended sediment was used to make smears on microscopic slides.

3.1.5. DIRECT MICROSCOPIC EXAMINATION:

Four thin smears on new and clean grease-free microscopic slides were made. By the use of sterile Pasteur Pipette, a drop of the sediment was spread

on each of the slide over an area of about 1 X 2cm. The drop was evenly spread over an area not exceeding the above size (Kent & Kubica 1985). The smears were allowed to air-dry and then heat-fixed.

Ziehl-Neelsen, (Z-N) Kinyoun modified AAFB and Gram's stainings were performed on the slides from each of the samples. For the Z-N staining, mixture of basic fuchsin and phenol crystal was used as the primary stain and counter stained with 0.1 per cent methylene blue (Cruickshank et al. 1975). In the Kinyoun cold-staining procedure, five per cent aqueous sulphuric acid was used in place of acid alcohol as the decolourizing agent (Cruickshank et al. 1975). Gram's staining technique was performed on the third slide (Runyon et al. 1974).

All the stained slides were examined under immersion oil. The entire smear on each slide was examined.

3.1.6. **CULTURAL PROCEDURES:** Each reconstituted sample was inoculated into the media listed below. This was by using sterile Pasteur pipette to inoculate two drops of the deposit into each medium in duplicate.

(a) Lowenstein-Jensen (L-J) slant with glycerine.

(b) Lowenstein-Jensen slant without glycerine

(c) Dubos oleic acid-dextrose catalase (OADC) agar slant,

(d) A plate of sabouraud dextrose agar and

(e) A plate of blood agar.

The slants and plates were incubatd at 37°C in a carbon dioxide incubator.

The caps of the tubes were kept loose for one day to permit exposure to carbon dioxide which stimulates growth of mycobacteria (Whitcomb et al. 1962; Kent & Kubica, 1985). During that period the tubes were kept in the incubator in a horizontal position to maintain the proper spread of the

inoculum. The caps were then tightened to prevent dehydration of the media, and the tubes were then arranged in test tube basket and kept in standing position.

The tubes were checked every day for growth for the first week of incubation, and three times a week thereafter for eight weeks, while the plates were checked every day for two days. Any growth was immediately stained by the ZN, Kinyoun modified AAFB and Gram's techniques. The contaminated cultures were discarded and the samples reprocessed. The concentration of sodium hydroxide was increased to six percent whenever subsequent contamination occured (Kent & Kubica, 1985).

3.1.7. EFFECT OF N-ACETYL-L-CYSTEINE-SODIUM HYDROXIDE (NALC-NaOH) ON THE MICROFLORA IN CLINICAL SAMPLES:

The effect of 4 per cent sodium hydroxide on the microflora in 120 of the clinical samples was studied by inoculating a loopful of each of the sample into sabourand dextrose and blood agar plates before and after exposure to sodium hydroxide decontaminant.

Sabourand dextrose and blood agar plates were aseptically inoculated with the freshly collected samples before decontamination with 4 per cent sodium hydroxide. The samples were then exposed to the decontaminant for 30 minutes and immediately neutralized using 8 per cent hydrochloric acid (Cruickshank et al. 1975). The deposit obtained after spinning at 3000r.p.m. for 20 minutes was used to inoculate fresh plates of Sabouraud dextrose and blood agar. The plates were incubated at 37°C and observed every day for growth. Any growth was immediately stained by the Z-N, Kinyoun modified and Gram's methods.

N-acetyl-L-cysteine hydrochloride, which is a mucolytic agent, functions as a digestant of mucopurulent samples. It has been shown to have no effect on microorganisms when they are exposed to it in the above manner (Kubica et al. 1963b).

3.1.8. **MAINTENANCE OF STOCK CULTURES:**

Isolates were tested for purity by the Z-N, Kinyoun modified method of acid-fast and Gram staining. When an isolate was confirmed to be acid-fast, it was subcultured onto a fresh L-J slant and incubated at 37°C. Immediately an obvious growth with discrete colonies was observed, the slants were transfered into a freezer for preservation. Viability of the isolates was tested for, every month for three months by subculturing onto L-J slant and incubating at 37°C. Subsequent subculture onto L-J slant was then made every three months thereafter (Wayne & Doubek, 1964).

The above method of maintaining the stock cultures was adopted after trying glycerol method of Tarshis (1960) by which seven of the isolates were lost after one month of preservation in freezing condition in glycerol broth.

3.1.9. **CHARACTERISATION PROCEDURES:**

Growth obtained in the primary isolation media were subjected to Z-N, Kinyoun modified and Gram's staining to test for purity. Once purity has been established, several colonies were aseptically picked and inoculated into 5ml. of Dubos broth in 16 X 125ml. screwcapped test tubes. These cultures were incubated at 37°C for seven days; shaking the cultures daily by hand to increase the amount of growth and to obtain an even suspension of the organism at the end of the incubation period. The cultures were used as inoculum for pigmentation test, speed of growth and biochemical tests.

3.1.9.1. PIGMENT PRODUCTION AND SPEED OF GROWTH:

These tests permit the classification of a particular isolate to the Runyon's subgroups (Kubica et al. 1963a; 1973). Four L-J slants were inoculated using the Dubos broth cultures as the inocula. Two of the L-J slants were wrapped in aluminium foil and the other two left unwrapped. The four tubes were incubated in a carbon dioxide incubator at 37°C. The unwrapped tubes were observed everyday for growth and pigmentation for the first week of incubation and three times per week thereafter. Once growth was recorded in any of the unwrapped tubes, the corresponding unwrapped tubes were removed from the aluminium foil and examined for growth and pigmentation. Wherever pigmentation was not observed in the wrapped tubes, they were exposed to light for 3-5 hours. The caps were kept loose, during the light exposure period (Wayne & Doubek, 1964). The aluminium foil was then replaced and the cultures were re-incubated at 37°C and examined after three days for pigmentation.

Observations of the isolates were recorded as follows:

(a) Any growth occuring within seven days of inoculation of the LJ slants was labelled as rapid grower (Runyon et al. 1974; Kubica et al. 1975).

(b) Any growth occuring after seven days were labelled as slow grower and further subgrouped into:

(i) Scotochromogens, that is, those pigmented both in the dark and in the light.

(ii) Photochromogens, that is, those pigmented (yellow-to-orange) only after light exposure. (Tsukamura, 1981).

(iii) Achromogens, that is, no pigment produced either in the dark or after exposure to light.

3.1.9.2 **DETECTION OF NIACIN PRODUCTION:**

The chemical method of detection of niacin in culture medium was employed for this test (Runyon et al. 1959; Gangadharam & Droubi, 1971). Cultures that were 3-4 weeks old on L-J medium were used. One millilitre of sterile, distilled water was aseptically added to the culture slants and the tubes placed horizontally so that the fluid covered the entire surface of the medium for niacin extraction. Extraction was allowed for 15 minutes. 0.5 ml. of the fluid was removed in a clean 16 X 125ml. screwcapped test tube. A 0.5ml of each of 4 per cent aniline and 10 per cent cyanogen bromide were added to the extracted fluid. Immediate formation of a yellow colour was read as positive for the production of niacin.

3.1.9.3. **NITRATE REDUCTION TEST:**

The classical method of using liquid reagents for the reduction of nitrate to nitrite was employed (Bonicke et al. 1970; Lampe, 1981; Warren et al. 1983). The culture slants used for the detection of niacin production were also used to inoculate 2.0ml of 0.01M sodium nitrate substrate in 0.022M phosphate buffer contained in a 16 X 125ml screwcapped tubes. The tubes were shaken to have an even suspension of the bacilli in the medium, after which they were incubated for two hours at 37°C. One drop of 50 per cent hydrochloric acid, two drops of 0.2 per cent sulfanilamide and two drops of 0.1 per cent N-naphthylethylenediamine dihydrochloride were respectively added to the culture medium. The tubes were examined immediately for a pink-to-red colour, which was read as positive for the test.

A tube containing sodium nitrate which was not inoculated with the organism was used as the control. Included in the control was the addition

to negative tubes of zinc dust to check whether the reduction has progressed beyond nitrite to nitrogen gas and water. If nitrate was present it would be reduced by the zinc, and a red colour would develop, indicating a true negative test. If on the other hand the reduction has gone beyond nitrite, there would be no colour change after the addition of zinc dust. The test was therefore repeated to confirm the false negative nitrate reduction test (Lampe, 1981).

3.1.9.4. **CATALASE PRODUCTION:**

Semiquantitative catalase technique was employed (Kubica et al. 1966 and Wayne & Doubek (1968). Lowenstein-Jensen medium dispensed in 5ml amount into a 20 X 150ml tubes and insipissated as butts were aseptically inoculated using 0.1ml of a 7-day old Dubos broth cultures of the test organism. The tubes were incubated with caps left loosely for 2-3 weeks at 37°C in CO_2 incubator. One mililitre of freshly prepared Tween-peroxide mixture was added to the tubes and allowed to stand for five minutes at room temperature. The height of the column of bubbles above the medium was then measured in milimetres with a plastic ruler. The test was read as positive for those producing more than 45mm of bubbles along the column of the tube; and as negative for those producing less than 45mm of bubbles. An uninoculated tube was used as a control.

3.1.9.5. **TWEEN HYDROLYSIS:**

The substrate that was used to test for Tween hydrolysis was made up of 100ml of phosphate buffer solution at PH 7.0 to which was added 0.5ml Tween 80 and 2.0ml aqueous solution of 0.1 per cent neutral red (Wayne et al. 1964; Kilburn et al. 1973). It was dispensed in 2.0ml amounts into a 16 X 125ml screw-capped tubes and sterilized by autoclaving.

The substrate was inoculated with a loopful of the test organism from an actively growing young culture in Dubos broth and incubated at 37°C in a carbon dioxide incubator. The tubes were examined after one, five and ten days for any colour change.

An uninoculated tube of the substrate was used for the colour control. Any colour change from amber colour of the substrate to pink or red was recorded as a positive Tween 80 hydrolysis. The number of days for the colour change was recorded.

3.1.9.6. **ARYLSULPHATASE PRODUCTION:**

The Wayne modification technique of 3-day test was employed (Wayne, 1961). A 65 mg of tripotassium phenolththalein disulfate salt and 1.0ml of glycerol was added to 100ml of Dubos agar. It was dispensed in a 2ml amounts into 16 X 125ml screw-capped tubes and autoclaved. The medium was allowed to solidify in an upright position. A turbid suspension of the test organism was prepared in sterile water. One drop of it was used to inoculate the butt of the medium. Inoculated tubes were incubated at 37°C for 3 days. One millilitre of 1M sodium carbonate solution (Na_2CO_3) prepared by ading 10.6g anhydrous Na_2CO_3 to 100ml of distilled water was added to the cultures. Colour change from colourless to red was read as positive test. An uninoculated substrate medium was used as a negative control.

3.1.9.7 **HEAT STABLE CATALASE TEST:**

Several colonies of the test organism were suspended in a 0.5ml phosphate buffer solution at P^H7 contained in a 16 X 125ml screw capped tube. The suspension was incubated in a water bath at 68°C for 20 minutes. The suspension was cooled to room temperature and 0.5ml of Tween-peroxide was added. Formation of bubbles in the tubes were read as a positive heat stable catalase test (Wayne and Doubek, 1968).

TABLE 3

KEY DIFFERENTIAL FEATURES OF MYCOBACTERIA RECOVERED FROM HUMANS

	Species of Mycobacteria																								
	TB Complex		Photochromogen				Scoto-Chromogen				Nonphotochromogen										Rapid Grower				
Test or Property	*M. tuberculosis*	*M. bovis*	*M. kansasii*	*M. marinum*	*M. asiaticum*	*M. simiae*	*M. scrofulaceum*	*M. szulgai*	*M. gordonae*	*M. flavescens*	*M. xenopi*	*M. avium*	*M. intracellulare*	*M. malmoense*	*M. ulcerans*	*M. haemophilum*	*M. gastri*	*M. terrae complex*	*M. triviale*	*M. fortuitum*	*M. chelonei*	*M. abscessus*	*M. smegmatis*	*M. vaccae*
Growth rate	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R
Growth temperature: 22°-25° C	—	—	+	+			+	+	+	+	—	F	M	+	—	+	+	+	+	+	+	+	+	+
32°-33° C	+	+	+	+	+		+	+	+	+	F	+	+	+	+	5	+	+	+	+	+	+	+	+
35°-39° C	+	+	+	3	+	+	+	+	+	+	+	+	+	+	—	–	+	+	+	–	+	+	–	+
41°-43° C	—	—				—			—		+	+	M	–	—	–	M	M	F	M	M	+	+	F
Niacin production	+	—	—	F	–	+	—	—	—	—	—	—	—	–	—	–	—	—	—	—	V	—	—	—
Nitrate reduction	+	—	+	—	–	—	—	+	—	+	—	—	—	–	—	–	—	+	+	+	—	+	+	+
Catalase (>45 mm foam)	—	—	+	—	+	+	+		+	+	—	—	—	–	+	–	—	+	+	+	+	+	+	+
Catalase after 68° C 20 min	—	—	+	F	+	+	+		+	+	+	+	+	+	+	–	—	+	+	+	+	+	M	+
Pigmentation in dark	—	—	—	—	–	—	+	4	+	+	2	—	—	–	—	–	—	—	—	—	—	+	—	V
Pigment photoactivated	—	—	+	+	+	+	2	2, 4	2	—	—	—	—	–	—	–	—	—	—	—	—	—	—	+
Tween hydrolysis (5 or 10 days)	F	—	+	+	+	—	—	F	+	+	—	—	—	+	—	–	+	+	+	M	—	+	+	+
Tellurite reduction (3 days)	—	—	—	—			—		—	F	—	M	M			–	—	—	—	M	F	M	+	M
Resistant 10µg TCH per ml	+	—	+	+	+	+	+		—	+	+	+	—	+		–	—	+	+	+	+	+	+	+
Growth in presence of 5% NaCl	—	—	—	—	–		—		—	+	—	—	—	–		–	—	+	+	+	M	+	+	+
Iron uptake (rusty colonies)	—	—	—	—			—		—	—	–	—	—		—		—	—	—	+	—	+	+	+
Arylsulfatase positive (3 days)	—	—	—	—	–		—	M	—	—	M	—	—		—		—	—	F	+	+	—	—	—
Growth on MacConkey agar	—	—	—	—			—		—	—	—	—	F				—	—	—	+	+	—	—	—
Urease activity	+	+	+				+		—	+	—	—	—	–		–	+	—		+	+			
Clinical significance	+	+	+	+		+	+	+	—	—	+	+	+	+	+	+	—	—	—	+	+	—	—	—

1. Key to symbols: S = slow, R = rapid, + = >84% of strains +, M = 50–84% of strains +, F = 16–49% of strains +, – = <16% of strains +, V = variable blank spaces = few or no data available.
2. Pigment intensifies either with age or after prolonged (2 weeks) exposure to light.
3. Strains exhibit growth at 37° C on primary isolation usually grow only at 31° to 33° C.
4. *M. szulgai* is photochromogenic when grown at 37° C but photochromogenic at 25° C.
5. Grows from 25° to 30° C, no growth at 31° C. Requires hemin for growth.

Adapted From ATS, (1981).

3.1.10. ISOLATES OBTAINED FROM FACULTY OF VETERINARY MEDICINE FROM PREVIOUS STUDY OF ACID-FAST BACILLI:

Eighteen isolates obtained from previous studies in the Faculty of Veterinary Medicine were stained by the Z-N staining technique, and for the presence of other organisms by the Gram's and Kinyoun modified acid-fast staining techniques. Where a mixed growth of acid-fast bacilli and other organisms occurs, decontamination using 4 per cent sodium hydroxide was performed to obtain pure cultures of acid-fast bacilli. Biochemical tests were then carried out on the acid-fast cultures.

3.2. TUBERCULIN TESTING OF CONTACTS AND CULTURAL EXAMINATION OF THOSE WHO WERE POSITIVE:

The tuberculosis annexe attends to two groups of patients viz those confirmed by previous test to have tuberculosis, and the other group consisted of their relations either complaining or showing symptoms of tuberculosis. The second group was classed as contacts. They were examined routinely for tuberculosis infection by means of Mantoux technique.

Pure protein derivative with batch number RT231180 was used for the tuberculin skin test. Using the Mantoux technique 0.1ml (5TU) was administered intradermally. The skin reaction was read after 48 hours (Wijsmuller, 1966). The contacts with an induration of 10mm and above were read as positive for the Mantoux test, while those below 10mm were considered as negaive test (ATS, 1981; Abrams & Schlaegel, 1983; Spinaci et al. 1989). One hundred and fifty patients that were considered as positive for the Mantoux test and without history of vaccination with BCG were selected for further study. They were asked to produce their sputum samples, which were examined by direct microscopy and cultural methods as described earlier.

3.3. THE ROLE OF NOCARDIA IN TUBERCULOSIS SUSPECTS:

3.3.1. PATIENTS:

The same patients were examined for nocardiosis in order to determine the role of *Nocardia* in patients suspectd to be suffering from tuberculosis.

3.3.2. ISOLATION TECHNIQUE:

One slant each of LowensteinJensen medium, and oleic acid-dextrose-catalase agar and a plate of sabouraud dextrose agar were inoculated with the sample deposit that was obtained after decontamination and concentration procedures. The harsh decontamination effect of sodium hydroxide is known not to kill *Nocardia* if the exposure time was not more than 30 minutes (Freeman, 1983). Inoculation was aseptically made into the above culture media, using a sterile Pasteur pipette to deliver two drops of the deposit. The tubes were incubated at 37°C in a horizontal position with the caps loosened for 24 hours to allow proper spreading of the inoculum and evaporation of excess fluid in the tubes. After that period the caps were tightened and the tubes kept in the incubator in an upright position. The inoculum was streaked on the plate by means of a sterile wire loop. The cultures were observed everyday for growth over a period of four weeks (Georg *et al*. 1961).

3.3.3. CHARACTERISATION:

3.3.3.1. Staining Characteristics:

Any growth obtained in the tubes and plates was immediately stained with the Z-N, Kinyoun modified acid-fast staining and Gram's techniques. Isolates that stained only with the ZN technique were presumptively labelled as *Mycobacterium* species. Those that stained with the Kinyoun modified technique and were Gram positive were tentatively labelled as *Nocardia*.

3.3.3.2. **Pellicle Formation:**

Thioglycollate broth was inoculated with a loopful of the sample deposit and incubated at 37°C. Pellicle formation on the surface of the broth is positive for nocardiae (Carter & Buhler, 1957).

3.3.3.3. **Paraffin Utilization:**

Paraffin utilization was tested according to the method of Georg *et al.* (1961). Glass rods coated with paraffin were placed in tubes containing 5ml of Czapek's fluid without carbonhydrate. Small portion of growth was used to inoculate the fluid by means of a sterile wire loop. The tubes were incubated at 37°C. Cultures were observed for evidence of growth on the paraffin at two days interval over a four-week period.

3.3.3.4. **Casein Hydrolysis:**

This was performed to separate *N. asteroides* and *N. brasiliensis*. A 10 per cent skim milk and equivalent volume of 2 per cent agar in distilled water, were mixed, and distributed in 10ml quantities into test tubes. It was autoclaved at 121°C for 5 minutes.

The tubes were inoculated with the test organism and incubated at 37°C. They were examained for evidence of hydrolysis, which is indicated by the liquefaction of the medium, between seven days and fourteen days of incubation (Gordon and Mildred, 1956 a).

3.4. **DETERMINATION OF DRUG SENSITIVITY:**

3.4.1. **PRINCIPLE:**

The simplified variant of the proportion method of Canetti *et al.* (1963) was employed. It was based on the assumption that in all cases of tuberculosis there are some bacilli that are resistant to

antibacillary drugs, and that in resistant cases, the proportion of such bacilli is considerably higher than in sensitive cases. The proportion method consists of calculating the proportion of resistant bacilli present in a case. Two different bacillary dilutions, a high and a low one, were inoculated on drug-containing and drug-free medium, in order to provide numerable colonies on both media. The ratio of the number of colonies obtained on the drug-containing medium to the number of colonies obtained on the drug-free medium indicates the proportion of the resistant bacilli present in the strain. Below a certain proportion, the strain is classified as sensitive and above, as resistant (Table 5).

3.4.2. <u>DRUGS AND CULTURE MEDIA</u>:

Standard Laboratory powdered forms of isoniazid, streptomycin, ethambutol, rifampicin, pyrazinamide and thiacetazone (Sigma Co. Ltd.) in Lowenstein - Jensen medium without potato starch was employed (Kent & Kubica, 1985). Solutions of the drugs (except rifampicin and thiacetazone whose solubilities are low in water) were prepared in distilled water to give the final concentrations in the medium (critical concentrations) as shown in Table 4 (Canetti et al. 1969). Rifampicin and thiacetazone were dissolved in 2.0ml ethylene glycol and were further diluted accordingly using distilled water. The control medium without the drugs were prepared at the same time as the drug-containing medium.

After the drug has been added to the medium, it was distributed in 16 X 125 ml screw - capped tubes, and coagulated in slanting position at 85°C for 50 minutes. The slopes were left at room temperature overnight to check for contamination, after which they were stored at 4°C.

TABLE 4

PREPARATION OF DRUG MEDIA FOR SENSITIVITY TESTS

Drug	Amount of drug Weighed into 10ml sterile water	Dilution I: 1 in 9ml of sterile water	Dilution II: 1 in 9ml of sterile water	Amount (ml) of drug solution added to 200ml medium	Final drug concentration (µg/ml)medium
Isoniazid	100mg(100,000 µg) to give 10,000 µg/ ml drug solution	1 ml of drug solution to give 1,000 µg/ml	1 ml added to 9ml sterile water to give 100 µg/ml	0.4	0.2
Streptomycin	125mg(100,000µg potency to 10,000 µg/ ml drug solution	1ml of drug solution to 1,000 µg/ml	-	0.4	2.0
Enthambutol	100mg(100,000 µg) to give 10,000 µg/ml drug solution	1ml of drug solution to give 1,000 µg/ ml	-	1.2	6.0
Rifampicin	100mg in 2ml Ethylene glycol added to 8ml sterile water to give 10,000 µg/ml drug solution	1ml of drug solution to give 1000 µg/ml		0.2	0.1
Pyrazinamide	500mg(500,000 µg) to give 50,000 µg/ml drug solution	1ml of drug solution to give 5,000 µg/ml	-	1.0	25.0
Thiacetazone	100mg(100,000 µg)in 2ml Ethylene glycol added to 8ml sterile water to give 10,000 µg/ml	1ml of drug solution to give 1,000 µg/ml	-	0.4	2.0

ADAPTED FROM KENT AND KUBICA, 1985; CANETTI et al. 1969

3.4.3. **INOCULUM:**

Several scrappings of the test organism were made into 5ml sterile water containing some glass beads in a 16 X 125ml screwcapped tube. The tube was gently shaken to homogenize the bacterial suspension. Larger particles were allowed to settle and the supernatant suspension withdrawn into a fresh sterile screwcapped tube. This was made to contain 1mg/ml of tubercle bacilli by adjusting the turbidity to that of a MacFarland No. 1 turbidity standard with sterile distilled water. (Kent & Kubica, 1985). The bacterial suspension was diluted to 10^{-2} and 10^{-4} by making serial tenfold dilutions in sterile distilled water. Using a sterile pipette; about 0.1ml of each of the dilution was inoculated into 2 slants of medium without drug and 2 slants of each drug-containing (Canetti et al. 1969).

The slants were incubated at 37°C with caps loose in a horizontal position, ensuring that the inoculum covers the entire surface of the slant. The caps were tightened after the liquid part of the inoculum had evaporated (24 - 48hours).

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3.4.4. **READING OF TESTS:**

The results were read for the first time on the 28th day (Canetti et al. 1969; Aziz et al. 1989). The average number of colonies obtained for the two control slopes indicates the number of viable bacilli contained in the inoculum.

The average number of colonies obtained for the drug containing slopes indicates the number of resistant bacilli contained in the inoculum. The ratio between the second figure and the first indicates the proportion of resistant bacilli existing in the strain. The proportions are expresssed in terms of percentage. Below a certain proportion (the critical proportion) the strain is classified as

sensitive and above, as resistant (see Table 5). Any strain showing a proportion of resistant bacilli equal or superior to that indicated in Table 5 is classified as resistant to the corresponding drug.

If the result of the reading made on the 28th day is "resistant" no further reading of the test for that drug was required, and the strain was classified as resistant. If the result at the 28th day is "sensitive" a second reading was made on the 42nd day, to give a definitive result. (Canetti et al. 1969).

Table 5

Critical Proportion For Drug Resistance

Drug	Critical Proportion for resistance (%)
Isoniazid	1
Streptomycin	10
Ethambutol	10
Rifampicin	1
Pyrazinamide	10
Thiacetazone	10

Adapted from Canetti et al. (1969).

CHAPTER FOUR

RESULTS.

4. RESULTS:

4.1. ISOLATES FROM TUBERCULOSIS PATIENTS:

A total of 300 samples were collected and processed over a period of 69 weeks. They consisted of 229 sputa, 26 urine, six peritoneal fluids, 32 pleural fluids, three pus, one bone marrow and three lymph nodes.

The patients were made up of 216 males and 84 females, whose ages ranged between eleven and 68 years. The smear and culture results are shown in Table 6, while the results of individual patient are shown in appendix 1.

A total of 134 isolates were cultured from the 300 samples. The isolates which are being preserved in a freezer can withstand this storage, method for three months. Table 7 shows the correlation between direct microscopic examination and culture. The direct microscopic examination has a total sensitivity of 47.33 per cent, while the culture method has 44.66 per cent.

Chromogenicity and speed of growth were used to initially group the isolates. The results of these are shown in Table 8.

Seventy eight of the isolates were found to belong to the tuberculosis complex, 17 were photochromogens, nine achromogens and 30 rapid growers (that is, showed apparent colonies within seven days of incubation), and none was scotochromogenic.

Seventy five out of the 78 isolates that were labelled as TB complex were identified as *M. tuberculosis*. They were slow growers, that is, showed visible colonies after seven days of incubation, produced niacin, showed a positive nitrate reduction and produced a foam column of less than 45mm in the semiquantitative test. The remaining three isolates were identified as *M. bovis* as they were negative for the above tests.

TABLE 6

SMEAR AND CULTURE

Clinical Samples	Number Collected	Positive by only direct Micros-copic examina-tion	Positive by culture only	Positive by both Smear and Culture	Total Positive for both	Negative by both
Sputum	229	24	14	98	136	93
Urine	26	1	1	5	7	19
Perito-neal Fluid	6	0	0	1	1	5
Pleural Fluid	32	3	1	10	14	18
Pus	3	0	2	0	2	1
Bone Marrow	1	0	1	0	1	0
Lymph Node	3	0	1	0	1	2
TOTAL	300	28	20	114	162	138

TABLE 7

CORRELATION BETWEEN DIRECT MICROSCOPY AND CULTURAL RESULTS

Clinical Sample	Number Collected	Positive by direct Micros-copy	Positive by Culture	% Sensitivity By Direct Microscopy	% Sensitivity By Culture
Sputum	229	122	112	53.28	48.91
Urine	26	7	6	26.29	23.22
Peritoneal Fluid	6	1	1	16.66	16.66
Pleural Fluid	32	13	11	40.64	34.43
Pus	3	0	2	0	66.66
Bone Marrow	1	0	1	0	100.00
Lymph Node	3	0	1	0	33.33
TOTAL	300	142	134	47.33	44.66

The photochromogens showed no pigmentation in the dark but yellow pigmented colonies were produced in the tubes that were incubated in the light or when the tubes that were incubated in the dark were exposed to light for about four hours. These isolates reduced nitrate, hydrolysed Tween 80 and produced a column of foam which was greater than 45mm in the catalase test, but were negative for niacin production. They were all identified as M. kansasii.

The nine achromogens in addition to being slow growers, that is, produced apparent colonies after seven days of incubation, did not produce pigment both in the light and in the dark. These isolates were negative for niacin production, they did not reduce nitrate, did not hydrolyse Tween 80 and did not produce any gas measurable by catalase semiquantitative test. Arylsulphatase production was also negative, but showed catalase activity after heating at 68°C for 30 minutes and they were all identified as M. avium-intracellulare complex. There was no pathogenicity test carried on them to differenciat into avium or intracellulare. The rapid growers were nonpigmented both in the dark and light and showed apparent macroscopic colonies within seven days of incubation.

They were all positive for nitrate reduction and were positive for semiquantitative activity on hydrogen peroxide and were negative for niacin production. Nine of the rapid growers showed positive for arylsulphatase production and were identified as M. fortuitum, while four were positive for Tween hydrolysis and were therefore identified as M. smegmatis. The rest 17 isolates did not match any species in the Group IV rapid growers of Mycobacterium. Kinyoun modified cold-staining was therefore performed on them using five per cent sulphuric acid for decolourization.

Eleven of the 17 isolates stained with this technique and they were suspected to be Nocardia. The isolates formed pellicles in thioglycollate broth, they utilized paraffin and were negative for casein hydrolysis. They were therefore identified as Nocardia species, while the remaining isolates were labelled unidentified acid-alcohol fast microorganisms.

TABLE 8

GROUPING OF ISOLATES INTO RUNYON'S SUBGROUPS:

Runyon's subgroup	TB complex	Photochromogens (Group I)	Scotochromogens (Group II)	Achromogens (Group III)	Rapid Growers (Group IV
Number of isolates	78	17	0	9	30

4.2. A BIOCHEMICAL STUDY OF ISOLATES OBTAINED FROM THE FACULTY OF VETERINARY MEDICINE (FVM) FROM PREVIOUS STUDY OF ACIDFAST BACILLI:

Eithteen isolates obtained from previous studies in FVM were found to consist of fourteen pure cultures of acid-fast bacilli, two mixed cultures of acid-fast bacilli and Gram positive cocci and two Gram positive bacilli. Pure cultures of acid-fast bacilli were obtained from the two mixed cultures after decontamination with 4 per cent sodium hydroxide.

Biochemicals of acid-fast bacilli showed that eight were M. bovis, three M. tuberculosis, three M. gordonae and two M. smegmatis. Nine of these isolates were from "Nono", five of which were M. bovis, two M. smegmatis and two M. gordonae.

Six isolates were from sputum samples that were collected from cattle rearers. Three of them were identified as M. tuberculosis, two as M. bovis and one as M. gordonae. There was only one isolate from sour milk, and it was identified as M. bovis. The procedures for the identification of these isolates were as earlier outlined.

M. gordonae which was not isolated in the study on tuberculosis patient is a scotochromogen. It was negative for niacin prodcution, arylsulphatase production and nitrate reduction, positive for semigquantitative catalase test and Tween hydrolysis. Table 9 shows the biochemical reactions and identification of these isolates.

4.3 EFFECT OF N-ACETYL-L-CYSTEINE-SODIUM HYDROXIDE (NALCNaOH) ON CLINICAL SAMPLES:

Table 10 shows the growth of microorganisms from 120 clinical samples before and after decontamination with sodium hydroxide.

TABLE 9
BIOCHEMICAL REACTIONS & IDENTIFICATION OF ISOLATES OBTAINED FROM FVM IN A PREVIOUS STUDY

Isolate Number	Z-N	Gram's	Kin-youn	Pigment Production	Speed of Growth	Niacin Production	Nitrate Reduction	Catalase >45mm	Tween Hydrolysis	Arylsulphatase Production	Heat Stable Catalase	Presumptive Identification by this study	Presumptive Identification by FVM
P1 3(1)	+	-	+	+	R*	-	-	+	+	-	+	M.gordonae	M. gastri
SK 2(3)1	+	-	+	-	R	-	-	+	+	-	-	M.smegmatis	M. smegmatis
HD (15)2	+	-	+	-	S*	-	-	-	-	-	-	M. bovis	M. bovis
SK2(3)2	-	+	-	-	R							G+ bacillus**	M. smegmatis
HD17 SPT	+	-	+	-	S	+	+	-	-	-	-	M.tubercu-losis	M.triviale
HD20 SPT	+	-	+	-	S	-	-	-	-	-	-	M. bovis	M. triviale
HD19 SPTR	+	-	+	-	S	-	-	-	-	-	-	M. bovis	M. scrofulace
SK2(5)	+	-	+	-	S	-	-	-	-	-	-	M. bovis	M. gastri
JJ SPT 2	+	-	+	+	S	+	+	-	-	-	-	M.tubercu-losis	M. gastri
HD19 SPTA	+	-	+	+	R	-	-	+	+	-	+	M. gordonae	M. gastri
WS2(2)	-	+	-	-	R							G+bacillus	M. flavescens
ZR 3(2)	+	-	+	-	S	+	+	-	-	-	-	M.tubercu-	M. gastri
SB 3(3)	+	-	+	+	R	-	-	+	+	-	-	losis	
												M.gordonae	M.gordonae
SB 3(3)	+	-	+	-	S	-	-	+	-	-	-	M.bovis	M.gordonae
SB 3(1)	+	-	+	-	S	-	-	-	-	-	-	M.bovis	M.scrofulaceu
SK 2(3)2	+	-	+	-	R	-	-	-	-	-	-	M.smegmatis	M.smegmatis
GW 3(8)	+	-	+	-	S	-	-	-	-	-	-	M.bovis	M.gordonae
SB 3(2)	+	-	+	-	S	-	-	-	-	-	-	M.bovis	M.smegmatis

* R = Rapid growth
S = Slow growth
** G + bacillus = Gram Positive bacillus

TABLE 10

Efficiency of NALC-NaOH*Decontamination on 120 Samples:

Sample	Culture colony count before Decontamination	Culture colony count after Decontamination	Efficiency (%)
97 Sputa	63 Staphylococcus sp: 42 Gram positive cocci (in pairs & Chains) 7 Gram negative bacilli 2 yeast cells.	4 Staphylococcus sp: 9 Gram positive bacilli 7 of which were Nocardia	90.80
15 Urine	8 Staphylococcus sp: 6 Gram negative cocci (in pairs) 1 Gram positive cocci (in chains) 1 yeast cell.	2 Staphylococcus sp:	86.6
3 Peritoneal Fluids	1 Staphylococcus sp:	No Growth	100
1 Pleural Fluid	No Growth	No Growth	100
3 Pus	3 Staphylococcus sp:	No Growth	100
1 Bone Marrow	No Growth	No Growth	100

* NALC - NaOH = N-acetyl-L-cysteine sodium hydroxide

Generally, growth was profuse on both blood and Sabouraud dextrose agar before the addition of the decontaminant. Growth was very scanty wherever it occured after the addition of the decontaminant, with the population of colonies usually between one and three.

4.4. CULTURAL EXAMINATIONS OF CONTACTS WHO WERE TUBERCULIN POSITIVE:

A total of 150 sputum samples were collectd from close contacts of tuberculosis patients. The contacts consisted of 78 females and 72 males whose age ranged between 9 months and 60 years. Appendix 2 shows the results of the microscopy and culture of the concentrated samples obtained from the Mantoux reactors (that is the people with induration of 10mm and above). The Mantoux readings ranged between 15mm and 35mm with one patient showing an ulcerated induration.

There were 12 (or 8%) isolates from the 150 samples. Biochemical tests were performed on the isolates as earlier outlined above. Eleven of the isolates were identified as M. tuberculosis while the remaining one was M. kansasii. The patient with the highest number of bacilli per field had a Mantoux reading of 20mm and the patient with the lowest number of bacilli per field had a Mantoux reading of 29mm.

There was no relationship between the Mantoux reaction and the presence of acid-fast bacilli in the concentrated smears or the culture. The size of the induration due to Mantoux test did not also determine the amount of bacilli present in the concentrated smears. One patient presented with an ulcerated induration and was negative for AFB in her sputum sample and culture. The relationships among Mantoux test, direct microscopy and culture are shown in appendix 2.

4.5. The Role of Nocardia in Tuberculosis Patients:

All the 300 samples that were collected from the chest annexe were examined for the presence of Nocardia species. A total of eleven isolates were obtained with growth occuring on all the three media that were used for primary isolation. The media were Lowenstein-Jensen medium, blood agar and Sabouraud dextrose agar. The isolates were Gram positive "mycelia". They stained with the Kinyoun modified acid-fast technique, employing five per cent aqueous sulphuric acid as the decolourizer. They formed pellicles in thioglycollate broth and utilized paraffin as a sole carbon source. None of the isolate was able to hydrolyze casein, indicating that all isolates were N. asteroides. Table 11 shows the physical and chemical characteristics of the isolates.

4.6.1 PRIMARY DRUG SENSITIVITY PATTERNS:

Table 12 shows the primary drug sensitivity patterns of 123 mycobacterial isolates. The isolates were obtained mostly from patients who reported with a history of newly diagnosed and no evidence of previous treatment. Seventeen of the patients had a history of treatment for less than 15 days with the drug(s) before the sample collection. The period was considered to be insufficient to influence the emergence of drug resistant strains. Resistant cultures from these patients were therefore of the primary type (Narain et al. 1968). However, there were twelve patients with reactivated disease. They had earlier received treatment and discharged from the clinic and because they had a relapse, reported back to the clinic. Resistant cultures from those patients were of the acquired type.

TABLE 11

PHYSICAL AND CHEMICAL CHARACTERISTICS

OF NOCARDIA ISOLATES

Sample Number	Growth Rate (days)	Gram's Reac- tion	Pellicle Forma- tion	Paraffin Utiliza- tion	Casein Hydro- lysis	Specie
35	2	+	+	+	-	N.asteroides
36	4	+	+	+	-	"
56	7	+	+	+	-	"
83	2	+	+	+	-	"
104	3	+	+	+	-	"
114	2	+	+	+	-	"
116	6	+	+	+	-	"
134	9	+	+	+	-	"
143	2	+	+	+	-	"
178	4	+	+	+	-	"
179	2	+	+	+	-	"

Critical proportions for resistance against isoniazid (INH), streptomycin, ethambutol, rifampicin, pyrazinamide and thiacetazone employing the simplified variant of the proportion method of Canetti et al. (1963) were recorded for each of the isolates. Table 12 shows the susceptibility patterns of 123 isolates including the unidentified isolates. Isolates from patients with a history of previous treatment were excluded from this Table. They consisted of eleven M. tuberculosis and one M. kansasii.

The results of the prevalence of the drug sensitivity are summarized in Table 13. Among the six drugs tested, resistance to isoniazid and streptomycin were the highest; 18.66% and 13.33% respectively for the M. tuberculosis isolates. The resistance of M. tuberculosis to thiacetazone and ethambutol were 9.33% and 6.66% respectively, while resistance to pyrazinamide and rifampicin were the lowest with 5.33% and 4.00% prevalence respectively.

Multiple drug resistance, that is, resistance to more than one drug was observed for some of the M. tuberculosis isolates. One of these was a case of resistance to four drugs, viz, isoniazid, streptomycin, pyrazinamide and thiacetazone. It was a case of renal tuberculosis on fresn therapy. The patient's father died from tuberculosis. It is therefore likely that she could have been infected with initially resistant strains. There were four (5.33%) isolates of M. tuberculosis that were resistant to two drugs. Two of them were pulmonary cases, while two were disseminated tuberculosis, from which isolations were made from the lymph nodes. One of the pulmonary cases was resistant to isoniazid and streptomycin, while the other was resitant to isoniazid and thiacetazone. The disseminated cases wee both resistant to streptomycin and thiacetazone. There were no cases of resistance of M. tuberculosis to five or six drugs.

TABLE 12

PRIMARY DRUG SENSITIVITY PATTERN
OF 123 MYCOBACTERIAL ISOLATES

Species	Isoniazid		Streptomycin		Rifampicin		Pyrazinamide		Ethambutol		Thiacetazone	
	S*	R*	S	R	S	R	S	R	S	R	S	R
M tuberculosis (75) **	61	14	62	13	72	3	71	4	70	5	68	7
M bovis (3)	3	0	3	0	3	0	0	3	3	0	3	0
M kansasii (17)	9	8	12	5	17	0	17	0	17	0	16	1
M avium complex (9)	1	8	1	8	9	0	0	9	9	0	8	1
M fortuitum (9)	0	9	0	9	9	0	9	0	9	0	9	0
M smegmatis (4)	3	1	3	1	4	0	2	2	3	1	3	1
Unidentified (6)	4	2	6	0	6	0	5	1	6	0	6	0

* S = Sensitive

R = Resistance

** Figures in parenthesis indicate the number of isolates.

TABLE 13

PREVALENCE OF PRIMARY DRUG RESISTANCE (%)

SPECIES	DRUGS					
	ISONIAZID	STREPTOMYCIN	RIFAMPICIN	PYRAZINAMIDE	ETHAMBUTOL	THIACETAZONE
M. tuberculosis	18.66	13.33	4.00	5.33	6.66	9.33
M. bovis	0	0	0	100	0	0
M. kansasii	47.05	29.41	0	0	0	5.88
M. avium complex	88.88	88.88	0	100	0	88.88
M. fortuitum	100.00	100.00	0	0	0	0
M. smegmatis	25.00	25.00	0	50.00	25.00	25.00
Unidentified	33.33	0	0	16.66	0	0

The three isolates of M. bovis were all sensitive to pyrazinamide and resistant to the other five drugs. Similarly, the nine isolates of M. fortuitum were sensitive to isoniazid and streptomycin and were resistant to the other four drugs.

Seventeen isolates of M. kansasii were all resistant to rifampicin, pyrazinamide and ethambutol. Resistance to isoniazid, streptomycin and thiacetazone were 8(47.05%), 5(29.41%) and 1(5.88%). The nine isolates of M. avium complex were all sensitive to rifampicin and ethambutol and were all resistant to pyrazinamide. There were 8(88.88%) isolates that were resistant to isoniazid, streptomycin and thiacetazone. The four isolates of M. smegmatis were sensitive to rifampicin and 2(50.00%) were resistant to pyrazinamide. One of the two isolates of M. smegmatis that was resistant to pyrazinamide also showed multiple resistance to isoniazid, streptomycin, ethambutol and thiacetazone.

All the six unidentifiable isolates were sensitive to streptomycin, rifampicin, ethambutol and thiacetazone. Two of them were resistant to isoniazid, this represents 33.33% resistance. Only one showed multiple resistnce to isoniazid and pyrazinamide.

4.6.1. Acquired Resistance:

Resuslts of resistance of Mycobacterium to antituberculosis drugs for 12 patients who had previous therapy are shown in Table 14. The isolates consist of eleven M. tuberculosis and one M. kansasii.

The highest resistance was to isoniazid among the eleven M. tuberculosis isolates. Three or 27.27% were found to be resistant to isoniazid, while the lowest resistance was to rifampicin, pyrazinamide and ethambutol with only one isolate being resistant.

TABLE 14

PATTERN OF DRUG REACTION OF ISOLATES FROM PATIENTS WITH ACQUIRED RESISTANCE

Species	Drugs											
	Isoniazid		Streptomycin		Rifampicin		Pyrazinamide		Ethambutol		Thiacetasone	
	S*	R*	S	R	S	R	S	R	S	R	S	R
M tuberculosis	8	3	9	2	10	1	10	1	10	1	9	2
	(27.27)**		(18.18)		(9.09)		(9.09)		(9.09)		(18.18)	
M kansasii	0	1	1	0	1	0	1	0	1	0	0	1
	(100)		(0)		(0)		(0)		(0)		(100)	

* S = Sensitive

* R = Resistant

** Figures in parenthesis represent per cent resistance

Resistance to both streptomycin and thiacetazone was 2(18.18%) of the M. tuberculosis. The only M. kansasii that was tested for acquired drug resistance was sensitive to streptomycin, rifampicin, pyrazinamide, ethambutol but resistant to isoniazid and thiacetazone.

Multiple drug resistance was also observed among the isolates that were examined for acquired resistance. One of these was a case of resistance to five drugs, except to ethambutol. The isolate was from a policeman with pulmonary tuberculosis which relapsed for the second time after three years of initial conversion. In addition to the six anti-tuberculosis drugs that were used in this study, he had previous therapy with thiazina and ethionamide. There was also a retreatment pulmonary case in which multiple resistance to three drugs were observed. The drugs were isoniazid, streptomycin and thiacetazone.

CHAPTER FIVE

DISCUSSION

5: DISCUSSION

Currently, there are 54 recognized species of mycobacteria, 25 have been implicated in mycobacteriosis (Kent & Kubica, 1985). In this study, a total of 135 mycobacterial isolates were encountered, 49 were species other than M. tuberculosis. This represents over 36 per cent of mycobacteria that were not M. tuberculosis. Studies from various parts of the world indicate that different species of opportunistic mycobacterial pathogens predominate as a cause of disease in different geographical regions (Kotian et al. 1981).

In Nigeria, various workers have independantly isolated atypical mycobacteria. Anyawale (1989) in addition to M. tuberculosis and M. bovis isolated M. avium complex from the nasal secretions of tuberculin positive cattle. Shehu (1988) in the studies of raw milk obtained from cattle Fulani around Zaria, isolated seventeen atypical mycobacteria. Alhaji (1976) and Beer & Davis (1965) also isolated mycobacteria belonging to groups III and IV. The isolation, of over 36% mycobacteria other than M. tuberculosis, and belonging to Groups I, III and IV from human in this study is an indication of the dangers posed by atypical mycobacterial infection in Nigeria. The infections due to these mycobacteria might have been overshadowed by the high incidence of the disease caused by M. tuberculosis in our society. This is more so when acid-fast bacilli seen only in direct smears are most often mistaken to be M. tuberculosis by both our laboratory diagnosticians and clinicians. The significance in epidemiology and true pathogenic potential of atypical mycobacteria therefore needs a further evaluation, and since differenciation of tuberculous from non-tuberculous mycobacteria can easily be made on cultures, preliminary classification using the Runyon's scheme is therefore suggested for the routine diagnosis of tuberculosis in our diagnostic laboratories. Each state capital should establish a tuberculosis diagnostic laboratory and such laboratories should be equipped to at least Level II mycobacteriology laboratory.

In addition, the establishment of a National Laboratory which should have facilities of a level III mycobacteriology laboratory is suggested. It will serve as a reference centre to the state mycobacterial laboratories.

It is worthy of note that M. smegmatis which is a rapid growing atypical Mycobacterium was isolated in this study. This specie according to Good (1985) is a saprophyte and has no clinical significance (ATS, 1981). Its isolation in "nono" by the Faculty of Veterinary Medicine, and also in this study further call for an investigation into its true pathogenic status. This is moreso when it was isolated alone from clinically ill patients and not in association with other mycobacteria.

Only eight of battery of tests could be used in this study. Inadequacy in the number of tests might have contributed to the high number of the unidentified isolates. It is however certain they were not M. tuberculosis because of their rapid growth rate and they did not exhibit both niacin production and nitrate reduction (Kent & Kubica, 1985). Furtheremore, M. avium and M. intracellulare have similar biochemical characteristics and differenciation of the two is determined pathogenically: M. avium is virulent for fowls while M. intracellulare is not. Similarly, M. tuberculosis and M. africanum have similar biochemical behaviours (Tripathy, 1981). M. africanum strains are heterogenous in their properties and are seen to bridge the evolutionary gap between human and bovine types (Yates et al. 1984). In any case the use of standard strain known as H32Rv which has a stable characteristics of the parent strain could have helped in the study of biochemical properties of tubercle bacilli. In addition, reference strains would have further helped in the identification of the species. They were not available for use for reasons of cost and transportation from abroad.

Some of the isolates tagged M. tuberculosis could actually be M. africanum in view of the geographical location of

Nigeria between Senegal and Central Africa where M. africanum shares properties between M. bovis in the case of isolates from Senegal and M. tuberculosis in those isolated from Central and East Africa. Nigeria is located about the middle of these two geographical points.

It is worthy of mention that from the results of microscoy and culture (Table 7), the culture technique was less sensitive than the smear method. Of the 300 samples that were examined, 142 (47.33%) were positive for smear while 134 (44.66%) cultures were isolated. In a similar experiment, Greenbaum et al. (1980) showed that culture technique was more than twice as sensitive as the smear method in the diagnosis of pulmonary tuberculosis in a Teaching Hospital, thus proving the expected higher efficiency of culture method over smear examination. The importance of culture technique in the confirmation of tuberculosis needs emphasis, its use in characterisation and drug sensitivity tests not withstanding. However, the low improvement of this technique over microscopy in this study, no doubt, lends support to the reliability of Ziehl - Neelsen staining technique as a useful diagnostic indicator in alerting the clinician to the possibility of acid-fast bacilli infection in patients.

The reasons for the lower sensitivity of culture over microscopy in this study might include, the probable inactivation of the bacilli by the drugs in patients who commenced therapy before being included into this study, and also the biostatic effect of sodium hydroxide decontaminant, for it has been shown to have a biostatic effect on mycobacteria (Runyon et al. 1974).

One of the striking findings in the aspect dealing with characterisation was the great difference in the speciation of the eighteen isolates that were provided by the Faculty of Veterinary Medicine. It may be noted that only five of the identifications match one another. This great discrepancy might be very difficult to explain particularly when the identifications were done in the same laboratory and with

the same sources of reagents, and more so when similar techniques were employed. The use of reference strains could have remedied this situation.

However, the isolation of M. gastri, M. triviale and M. flavescens from sputum of apparently ill patients by the Faculty of Veterinary Medicine (FVM) raises serious doubt, because these are strict saprophytes (Kent & Kubica, 1985; Kotian et al. 1981; Kubica & Silcox, 1973; Kubica et al. 1973). The identification techniques by FVM did not include arylsuslphatase and heat-stable catalase in its battery of tests. This could also account for the differences in characterisation.

Though patients with BCG scar were excluded from the study dealing on the laboratory diagnosis of tuberculin reactors, it could have a bias effect on the results because it has been shown to wane (decay) with time (Fine, 1988; Pahano et al. 1982). However, the use of BCG scar in screening for patients who have had BCG has been shown to be reasonable in the absence of any other criterion (Spinaci et al. 1989).

The findings by direct microscopic and cultural techniques of 8 per cent tuberculosis among tuberculin reactors are reasonable when compared to that of Styblo & Meijer (1976), who obtained 7.8 per cent in a related study.

One important factor in this study is the 10mm "reference point" of induration that was arbitrarily chosen in an attempt to separate significant ('positive') from non-significant ('negative') reactions, because tuberculin test has been shown to have a regional variation (Comstock & Woolpert, 1978; Pust & Erickson, 1989) and therefore the interpretation of a tuberculin skin test should be guided by the purpose for which the test is given to limit the consequences of false classification (ATS, 1981).

However, Pust & Erickson (1989) and Spinaci et al. (1989) showed that the 10mm 'reference point' was reasonable unless the investigation is based on a regional study of the relative

likelihood of tuberculous or non-tuberulous infection in a specific population. In any case, sputum examination of household contacts is not a very good measure of infectivity, because those infected might have not developed the disease at the time of examination. Therefore, the fact that there was no correlation between culture positivity and tuberculin reaction size in contacts of tuberculosis patients is not suprising. This is because those with larger size reaction are relatively immune and can curtail multiplication of tubercle bacilli.

In this regard, tuberculin skin test remains a tool for screening infection. However, the inclusion of microscopy and culture techniques in a high risk group like the contacts of tuberculosis patient is suggested. This will enhance their early diagnosis and proper antituberculosis management.

Nocardia are Gram positve and are easily decolourized by the Ziehl-Neelsen technique (Freeman, 1983). These techniques are routinely employed in our hospitals, nocardiosis patients are therefore exposed to the dangers of being diagnosed as habouring Gram positive bacilli or even diagnosed as tuberculosis. It is therefore advised that Kinyoun modified acid-fast method be included in the routine laboratory examinations of tuberculous suspected patients. In addition, pellicle formation on the surface of thioglycollate broth is positive for nocardiae and in most cases growth occured between 2-3 days of inoculation (Carter & Buhler, 1957). The growth rate of Nocardia species that were isolated in this study range from 2-9 days. The delay in growth of some of the isolates might be attributed to the number of culturable bacilli that were present in the inoculum, and to some extent the biostatic effect of the sodium hydroxide which was used to decontaminate the samples.

Over the years a number of publications have appeared in which prevalence of resistance of M. tuberculosis to antituberculosis drugs were reported (Gangadharam, 1981; Mitchison, 1984; Aziz et al. 1989; Al-orainey et al. 1989).

The findings in this work of 18.66% primary resistance and 27.27% acquired resistance of M. tuberculosis to isoniazid; 13.33% and 18.18% of primary and acquired resistance to streptomycin, the two most commonly used drugs, are comparable to those from Europe, U.S.A, some Asian and African countries (Gangadharam, 1981).

The resistances reported in these countries range from 1.3% in Great Britain, 5% in U.S.A, 8% in East Africa to as high as 18.4% in Ghana, 20% in Bombay (India) and 23% in North Sudan. It is worthy to note here that countries with less than 10 per cent drug reistance belonged to the "developed countries", whereas above 10 per cent are those from the so called "third world" and Nigeria is one of such countries form the third world. Other studies have also shown that the percentage of primary resistance should be less than in acquired resistance, as is the case in this study. (Narain et al. 1968). In any case the very existence of drug-resistant strains of tubercle bacilli in any number constitutes a threat to the community in which patients with active tuberculosis remain undetected. Such organisms are a hazard in areas in which close contacts and living conditions may allow easy spread of infection and in areas in which a vast majority of persons are uninfected and thus normally susceptible to the disease.

It is worth suggesting here that in a future survey of drug-resistance among mycobacteria, consideration should not be given to the history of patient therapy. This is referred to as initial drug resistance (Citron, 1988). It includes both primary as well as acquired resistance; for example acquired resistance in patients who have either concealed a history of previous therapy or have received chemotherapy, for example isoniazid for a cough, without being aware of the medicament.

It takes elaborate efforts to check conclusively and determine the possibility of previous but unreported treatment. However, the 55 per cent incidence of defaulters and 74 per

cent of those who received inadequate chemotherapy among the 961 pulmonary tuberculosis patients reported by Lege (1980) in Zaria, requires that we know the amount of primary and acquired resistances. This will help in taking appropriate measures to reducing the instances of defaulters. Initial drug resistance will act as a guide to the formulation of chemotherapy programmes and reflect the quality and extent of chemotherapy in an area like ours where there is no carefully monitored case findidng followup or drug-treatment programme. Patients may choose to go to a hospital, local dispensaries, specialized tuberculosis treatment centres or to a private practitioner, trained or untrained. Thus, it is difficult to collect medical records, to establish an accurate history of treatment, or to establish the proportion of new cases who have drug resistance.

The isolation of atypical mycobacteria and Nocardia asteroides calls for caution in the interpretation of acid-fast bacilli seen in direct smears, because from this work, it is observed that not all acid-fast bacilli seen by direct microscopy are M. tuberculosis. It also calls for care in the formulation of chemotherapeutic regimen for the individual tuberculosis patient in our hospitals since antituberculosis drugs have been reported by Kotian et al. (1981) not to affect Nocardia.

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7. APPENDIX

Appendix 1: Biochemical Tests on Isolates From Tuberculosis Patients:

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin Production	Nitrate reduction	Catalase (>45mm)	Tweeen Hydrolysis	Arylsulphatase Producc-	Heat Stable Catalase	
1	Pink	14	-	-	-	-	-	-	M. bovis
3	Orange	13	+	+	-	-	-	-	M. tb.*
4	Achromogen	6	-	+	+	+	-	-	M. smegmatis
6	Orange	14	+	+	-	-	-	-	M. tb.
7	Orange	13	+	+	-	-	-	-	M. tb.
9	Achromogen	3	-	+	+	-	+	+	M. fortuitum
10	Achromogen	5	-	+	+	-	+	+	M. fortuitum
12	Orange	13	+	+	-	-	-	-	M. tb
16	Achromogen	4	-	-	-	-	-	-	Unidentified
19	Achromogen	17	-	-	-	-	-	+	M. avium cplex
24	Achromogen	4	-	-	-	-	-	-	Unidentified
26	Achromogen	9	-	-	-	-	-	+	M. avium cplex
27	Photochromogen	17	-	+	+	+	-	+	M. kansasii
29	Orange	19	+	+	-	-	-	-	M. tb.
30	Orange	16	+	+	-	-	-	-	M. tb
31	Orange	11	+	+	-	-	-	-	M. tb
32	Achromogen	4	-	+	+	+	-	-	M. smegmatis
33	Orange	16	+	+	-	-	-	-	M. tb
35	White	2	-	-	-	-	-	-	Nocardia
36	Colourless	2	-	-	-	-	-	-	Nocardia

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin production	Nitrate Reduc-	Catalase (≥45mm)	Tween Hydrolysis	Arylsulphataase Production	Heat Stable Catalase	
37	Orange	14	+	+	-	-	-	-	M. tb.
39	Yellow	14	-	-	-	-	-	-	M. bovis
41	Orange	17	+	+	-	-	-	-	M. tb
42	Achromogen	8	-	-	-	-	-	+	M. avium cpl
43	Achromogen	5	-	+	+	-	+	+	M. fortuitum
46	Orange	12	+	+	-	-	-	-	M. tb
48	Orange	11	+	+	-	-	-	-	M. tb
49	Achromogen	4	-	-	-	-	-	-	M.avium cple
51	Orange	13	+	+	-	-	-	-	M. tb
52	Orange	17	+	+	-	-	-	-	M. tb
53	Orange	13	+	+	-	-	-	-	M. tb
54	Orange	9	+	+	-	-	-	-	M. tb
55	Achromogen	13	-	-	-	-	-	+	M. avium cpl
56	Yellow	2	-	-	-	-	-	-	Nocardia
60	Achromogen	13	-	-	-	-	-	+	M. avium cpl
61	Orange	11	+	+	-	-	-	-	M. tb
63	Achromogen	9	-	-	-	-	-	+	M. avium cple
64	Photochromogen	15	-	+	+	+	-	+	M. kansasii
65	- " -	8	-	+	+	+	-	+	M. kansasii

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin Production	Nitrate Reduction	Catalase (>45mm)	Tween Hydrolysis	Arylsul Phatase Production	Heat Stable Catalase	
67	Orange	15	+	+	-	-	-	-	M. tb
68	Achromogen	4	-	+	+	-	+	+	M. fortuitum
69	Orange	11	+	+	-	-	-	-	M. tb.
71	Photochromogen	9	-	+	+	+	-	+	M. Kansasii
78	Orange	15	+	+	-	-	-	-	M. tb.
79	Colour less	11	+	+	-	-	-	-	M. tb.
81	Orange	13	+	+	-	-	-	-	M. tb.
83	Orange	2	-	-	-	-	-	-	Nocardia
85	Orange	19	+	+	-	-	-	-	M. tb.
104	Orange	2	-	-	-	-	-	-	Nocardia
108	Orange	17	+	+	-	-	-	-	M. tb.
109	Yellow photochromogen	11	-	+	+	+	-	+	M. Kansasii
110	Achromogen	6	-	-	-	-	-	-	Unidentified
111	Orange	17	+	+	-	-	-	-	M. tb.
113	Orange	22	+	+	-	-	-	-	M. tb.
114	Orange	4	-	-	-	-	-	-	Nocardia
116	Yellow	2	-	-	-	-	-	-	Nocardia
117	Photochromogen	16	-	+	+	+	-	+	M. Kansasii

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin Production	Nitrate Reduction	Catalase (>45mm)	Tween Hydrolysis	Arylsul Phatase Production	Heat Stable Catalase	
122	Photochromogen	9	-	+	+	+	-	+	M. Kansasii
123	Orange	16	+	+	-	-	-	-	M. tb.
127	Achromogen	3	-	+	+	-	+	+	M. fortuitum
128	Orange	11	+	+	-	-	-	-	M. tb.
129	Colourless	17	+	+	-	-	-	-	M. tb.
131	Achromogen	6	-	+	+	-	+	+	M. fortuitum
133	Orange	13	+	+	-	-	-	-	M. tb.
134	Orange	4	-	-	-	-	-	-	Nocardia
135	Orange	12	+	+	-	-	-	-	M. tb.
136	Achromogen	9	-	-	-	-	-	-	M.avium cplex
142	Achromogen	12	-	-	-	-	-	+	M. avium cplex
143	Orange	3	-	-	-	-	-	-	Nocardia
145	Orange	19	+	+	-	-	-	-	M. tb.
146	Orange	23	+	+	-	-	-	-	M. tb.
150	Colourless	16	+	+	-	-	-	-	M. tb.
153	Orange	13	+	+	-	-	-	-	M. tb.
154	Orange	27	+	+	-	-	-	-	M. tb.
155	Orange	12	+	+	-	-	-	-	M. tb.
156	Colourless	13	+	+	-	-	-	-	M. tb.
157	Photochro-	17	-	+	+	+	-	-	M. tb.

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin production	Nitrate Reduction	Catalase (≯45mm)	Tween Hydrolysis	Arylsul Phatase Production	Heat Stable Catalase	
159	Orange	9	+	+	-	-	-	-	M. tb.
162	Photochromogen	12	-	+	+	+	-	+	M. Kansasii
164	Orange	9	+	+	-	-	-	-	M. tb.
166	Orange	19	+	+	-	-	-	-	M. tb.
173	Orange	24	+	+	-	-	-	-	M. tb.
174	Achromogen	6	-	-	-	-	-	-	Unidentified
175	Orange	22	+	+	-	-	-	-	M. tb.
177	Orange	16	+	+	-	-	-	-	M. tb.
178	Orange	3	-	-	-	-	-	-	Nocardia
179	Yellow	5	-	-	-	-	-	-	Nocardia
183	Orange	17	+	+	-	-	-	-	M. tb.
184	Achromogen	6	-	+	+	-	+	+	M. fortuitum
192	Photochromogen	26	-	+	+	+	-	+	M. Kansasii
193	Achromogen	5	-	-	-	-	-	-	Unidentified
195	Orange	28	+	+	-	-	-	-	M. tb.
198	Orange	17	+	+	-	-	-	-	M. tb.
199	Pick	28	-	-	-	-	-	-	M. bovis
200	Photochromogen	15	-	+	+	+	-	+	M. Kansasii
201	Achromogen	5	-	+	+	+	-	-	M. smegmatis
205	Photochromogen								

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin Production	Nitrate Reduction	Catalase (745mm)	Tween Hydrolysis	Arlysul Phatase production	Heat Stable Catalase	
206	Orange	27	+	+	-	-	-	-	M. tb.
208	Achromogen	4	-	+	+	-	-	-	M. fortuitum
209	Photochromogen	13	-	+	+	+	-	+	M. Kansasii
212	Achromogen	4	-	+	+	+	-	-	M. Smegmatis
215	Orange	13	+	+	-	-	-	-	M. tb.
216	Orange	16	+	+	-	-	-	-	M. tb.
222	Orange	21	+	+	-	-	-	-	M. tb.
223	Orange	11	+	+	-	-	-	-	M. tb.
224	Orange	23	+	+	-	-	-	-	M. tb.
225	Yellow	29	+	+	-	-	-	-	M. tb.
226	Colourless	27	+	+	-	-	-	-	M. tb.
227	Orange	14	+	+	-	-	-	-	M. tb.
233	Orange	19	+	+	-	-	-	-	M. tb.
237	Orange	25	+	+	-	-	-	-	M. tb.
241	Orange	17	+	+	-	-	-	-	M. tb.
243	Colourless	4	-	-	-	-	-	-	Unidentified
244	Orange	16	+	+	-	-	-	-	M. tb.
246	Orange	21	+	+	-	-	-	-	M. tb.
247	Orange	13	+	+	-	-	-	-	M. tb.
251	Orange	17	+	+	-	-	-	-	M. tb.
254	Photochromogen								

Mycobacteriology laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin Production	Nitrate reduction	Catalase (745mm)	Tween Hydrolysis	Arlysul Phatase Production	Heat Stable Catalase	
255	Achromogen	6	-	+	+	-	+	+	M. fortuitum
256	Orange	15	+	+	-	-	-	-	M. tb.
257	Orange	13	+	+	-	-	-	-	M. tb.
259	Orange	22	+	+	-	-	-	-	M. tb.
260	Photochromogen	18	-	+	+	+	-	+	M. Kansasii
262	Orange	29	+	+	-	-	-	-	M. tb.
265	Photochromogen	16	-	+	+	+	-	+	M. Kansasii
S5	Photochromogen	22	-	+	+	+	-	+	M. Kansasii
S11	Orange	17	+	+	-	-	-	-	M. tb.
S12	Orange	29	+	+	-	-	-	-	M. tb.
S13	Orange	32	+	+	-	-	-	-	M. tb.
S20	Orange	19	+	+	-	-	-	-	M. tb.
S22	Orange	17	+	+	-	-	-	-	M. tb.
S27	Orange	22	+	+	-	-	-	-	M. tb.
S29	Orange	37	+	+	-	-	-	-	M. tb.
S30	Orange	26	+	+	-	-	-	-	M. tb.

* M.tb. = M. tuberculosis

Mycobacteriology Laboratory Number	TESTS								Species
	Pigmentation	Speed of Growth (days)	Niacin Production	Nitrate Reduction	Catalase (745)	Tween Hydrolysis	Arlysulphatase Production	Heat Stable Catalase	
S27	Orange	22	+	+	-	-	-	-	M. tb.
S29	Orange	37	+	+	-	-	-	-	M. tb.
S30	Orange	26	+	+	-	-	-	-	M. tb.

* M. tb. = M. tuberculosis

APPENDIX 2: RELATIONSHIP AMONG MANTOUX

TEST RESULTS DIRECT MICROSCOPY AND CULTURAL EXAMINATION

SAMPLE NUMBER	MANTOUX REACTION (mm)	MICROSCOPY	CULTURE
C_1	15	Negative	Negative
C_2	15	"	"
C_3	23	"	"
C_4	25	"	"
C_5	20	"	"
C_6	20	"	"
C_7	25	"	"
C_8	23	Positive	*M. tuberculosis*
C_9	25	Negative	Negative
C_{10}	20	"	"
C_{11}	25	"	"
C_{12}	18	"	"
C_{13}	20	"	"
C_{14}	23	"	"
C_{15}	25	"	"
C_{16}	29	Positive	*M. kansasii*
C_{17}	21	Positive	*M. tuberculosis*
C_{18}	18	Positive & greater than 20 per field	*M. tuberculosis*
C_{19}	22	Negative	Negative
C_{20}	20	"	"

SAMPLE NUMBER	MANTOUX REACTION (mm)	MICROSCOPY	CULTURE
C_{21}	30	Negative	Negative
C_{22}	18	"	"
C_{23}	22	"	"
C_{24}	28	"	"
C_{25}	29	"	"
C_{26}	27	"	"
C_{27}	12	"	"
C_{28}	11	"	"
C_{29}	17	"	"
C_{30}	16	"	"
C_{31}	22	"	"
C_{32}	19	"	"
C_{33}	22	"	"
C_{34}	20	"	"
C_{35}	10	"	"
C_{36}	19	"	"
C_{37}	19	"	"
C_{38}	25	"	"
C_{39}	35	"	"
C_{40}	20	Positive & Greater than 40 per field	M. tuberculosis
C_{41}	18	Negative	Negative
C_{42}	20	"	"
C_{43}	17	"	"
C_{44}	22	"	"
C_{45}	25	"	"

SAMPLE NUMBER	MANTOUX REACTION (mm)	MICROSCOPY	CULTURE
C_{46}	20	Negative	Negative
C_{47}	30	"	"
C_{48}	24	"	"
C_{49}	17	"	"
C_{50}	14	"	"
C_{51}	20	"	"
C_{52}	23	"	"
C_{53}	12	"	"
C_{54}	14	"	"
C_{55}	16	"	"
C_{56}	11	"	"
C_{57}	11	"	"
C_{58}	36	"	"
C_{59}	21	"	"
C_{60}	18	Positive	M. tuberculosis
C_{61}	17	Positive	M. tuberculosis
C_{62}	14	"	"
C_{63}	12	"	"
C_{64}	14	"	"
C_{65}	20	"	"
C_{66}	20	"	"
C_{67}	14	"	"
C_{68}	26	"	"
C_{69}	17	"	"
C_{70}	18	"	"
C71	13	Negative	Negative

SAMPLE NUMBER	MANTOUX REACTION (mm)	MICROSCOPY	CULTURE
C_{72}	12	Negative	Negative
C_{73}	18	"	"
C_{74}	22	"	"
C_{75}	24	"	"
C_{76}	21	"	"
C_{77}	15	"	"
C_{78}	18	"	"
C_{79}		"	"
C_{80}	25	Pošitive	M. tuberculosis
C_{81}	18	Negative	Negative
C_{82}	19	"	"
C_{83}	12	"	"
C_{84}	20	"	"
C_{85}	23	"	"
C_{86}	16	"	"
C_{87}	16	"	"
C_{88}	20	Positive	M. tuberculosis
C_{89}	22	Negative	Negative
C_{90}	17	"	"
C_{91}	16	"	"
C_{92}	20	"	"
C_{93}	15	"	"
C_{94}	15	"	"
C_{95}	16	"	"
C_{96}	22	"	"
C_{97}	18	"	"

SAMPLE NUMBER	MANTOUX REACTION (mm)	MICROSCOPY	CULTURE
C_{125}	17	Negative	Negative
C_{126}	10	"	"
C_{127}	18	"	"
C_{128}	15	"	"
C_{129}	17	"	"
C_{130}	22	"	"
C_{131}	13	"	"
C_{132}	17	"	"
C_{133}	15	"	"
C_{134}	22	"	"
C_{135}	18	Positive	M. tuberculosis
C_{136}	30	Negative	Negative
C_{137}	20	"	"
C_{138}	27	"	"
C_{139}	19	"	"
C_{140}	23	"	"
C_{141}	22	"	"
C_{142}	25	"	"
C_{143}	18	"	"
C_{144}	18	"	"
C_{145}	27	"	"
C_{146}	12	"	"
C_{147}	30	"	"
C_{148}	25	"	"
C_{149}	15	"	"
C_{150}	20	"	"

* Strongly positive = Skin reaction greater than 10mm and ulcerated.