CHAPTER - II

REVIEW OF LITERATURE
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(i) Safety in Laboratory:

Work-related infection are a recognised hazard for personnel employed in laboratory where infectious disease agents are handled (Pike 1976). Studies have shown the risk of tuberculosis infection to be three to five time greater for the mycobacteriology laboratory worker than for other personnel (Secretaries, Maintenance worker etc) in the same institute (Reid 1957, Harrington and Shannon 1976).

Most infectious in the mycobacteriology laboratory can be attributed to the unrecognised production of potentially infectious aerosols containing acid fast bacilli (Pike 1976). Although some aerosolised droplets are large (>5um) and settle rapidly to contaminate skin, clothing, and counter tops, the most dangerous aerosols are those that produce droplet nuclei, tiny dry particle <5um in size (that may contain 1 or 2 viable mycobacteria) that remain suspended almost indefinitely in air unless they are removed by controlled airflow or ventilation (Foord and Lidwell 1975).

Laboratories should be designated with proper ventilation and exhaust system. Kent and Kubica discuss a recommended layout for the mycobacteriology laboratories.

All laboratories must have a certified BSC (Biological safety Cabinet) (plate-1 A). These units must be maintained according to the manufacturers guidelines and all manipulations of infected material or viable cultures must be handled in this cabinet. Before and after use, the BSC working surfaces must be wiped with appropriate germicides. In addition, a germicide-soaked gauze or
towel should cover the works are, excluding airflow grids, during specimen processing. Effective germicides include Amyl or other phenol-soap mixtures 3% to 8% formaldehyde, 0.05% to 0.5% sodium hypochlorite (Concentration varies according to nature of contaminated surface; See NCCI.S Document M29- T2, 1991) 70% ethanol and 5% phenol.

Since no BSC is 100% effective and both physical and mechanical failure do occur, protective clothing (especially the face mask designed to filter >90% of particles ranging from 0.5um to 1.0n um. To optimised filtration efficiency of mask (and increase personnel protection) use metal nose tabs and masks ties to ensure a light fit on the face.

Hospital surgical gowns protect the skin and clothing from large droplets greater then 5um that may accidentally be splattered. Rubber gloves guard against infection through cuts or abrasions on the hands.

Autoclavable or disposable shoe covers and caps minimise the chance of transporting infectious agents from the isolation room to other parts of the laboratory even to your residence.

All ppd. negative pathology staff should be tested at least annually to detect a new infection in time to prevent development of disease by giving isonizid therapy.

It is common for ppd. negative hospital employees to tested regularly with ppd. for the physician, surgeons, anaesthesiologist and other who technically are not hospital employee are not be included. How ever in this date of risk of infection from some one with tuberculoses due to drug resistant organism. It is
important for all such persons to be included in tuberculosis surveillance. After all TB is an equal opportunity infection.

A careful development and implemented infection control policy and appreciation of the concept of universal precautions is fundamental for the protection of all laboratory personal especially those working in mycobacteriology. Central to such policy is an annual health assessment for each employee. In addition mycobacteriology laboratories workers should be required to wear protective garb and perform all tasks with specimen or cultures in a properly functioning biological safety cabinet under negative pressure to the laboratory or in a centrifuge with containers that are shielded to prevent leakage. It should be intrinsic in all activities to guard against the generation of aerosols that may contain infectious particles. Rigorous attention to the decontamination of work areas and general order lines of the laboratory and a detailed understanding of mycobacteriology laboratory design and safety are critical to the protection of personal (Kent & Kubica, 1985).

Precautions that should be taken by laboratory workers against the infection with the tubercle bacilli's were listed in the early 1950 in the USA by Fish and Spendlove (1950) and by Long (1951). It was recognised at the time however that the long incubation period of the disease and exposure to the organism outside the laboratory made it difficult to determine if infection were job associated.

Reid (1957) first quantified the incidence of the disease among the staff of the clinical laboratory in the UK. He compared it with that of groups of post office staff and found that laboratory workers were from two to five time more likely to contract the disease than the matched controlled he identified 151 case of
tuberculosis among a total laboratory staff of 4824 in 368 laboratories during the year 1949-53. But excluded 51 who might not acquired the disease in the laboratory.

It is interesting to compare Reid's findings with those obtained some year later by Harington and Shannon (1976). Who confirmed their investigations to single year 1971 in England and 1973 in Scotland. When they compared their figure with those for the general population they found that the risk of the laboratory workers was five times greater than that to the community at large. There appeared to have been little improvement in UK in 15 or 16 year in spite of recognition of the problem by the health authority and the publication by them of excellent recommendation for the safe handling of tuberculous material (report 1958, department and social security 1770). The problem persisted and however responsibility for not following the officials recommendations must be shared by both employers and employees.

Eventually a code of practice for the prevention of infection in clinical laboratories (Department of Health and Social Security, 1978) was produced and the implementation of its requirement are monitored by a new statutory bodies the Health and safety Executive.

Such was the situation in the UK other countries have also had problems and some have found ways of dealing with them Collins (1982,1983).

Perform all procedures, including processing of specimens, smear preparation, inoculum preparation, making dilutions, inoculations of media, subculturing, etc., in a suitable biological safety cabinet (plate-1 A) in a room with an appropriate ventilation system as recommended by CDC (Kent and Kubica
1985; Vestel 1975). Use proper protective gown, mask, and gloves while handling specimens and cultures of potential pathogens. Follow CDC and OSHA recommendations.

The rubber septum of a vial should be disinfected each time the vial is entered. Clean the top of each vial with disinfectant followed by 70% alcohol or 5% phenol solutions.

Use an appropriate disinfectant for cleaning the work area and swabbing the vial septum and other surfaces. Phenol is no longer recommended as a disinfectant due to the documented toxicity of this compound to personnel. Other phenolic derivatives such as Amphi®, Staphene®, etc. are available, and should be substituted for 5% phenol. The CDC states: “With so many disinfectant available, it is important to consult the product brochures to make certain that the disinfectant is bactericidal for mycobacteria” (Kent and Kubica 1985).

Working with a syringe and needle requires special attention and safety precautions. Use syringes with permanently attached needles. Handle the syringe very carefully. Do not put the cover of the needle back after use. Discard syringes in sharp containers and handle the disposal containers very carefully. Refer to NCCLS Proposed Guideline H17P, “Protection of Laboratory Workers from Instrument Biohazards”.

Gas production by mycobacteria is not enough to build up positive pressure inside the 21B medium vial. However, contaminating bacteria may cause such a positive pressure build-up. Vent all positive culture vials to release gas before sampling for subculturing or staining. Venting and sampling should be performed
in a biological safety cabinet, with appropriate protective clothing, including gloves and masks.

Prior to use, examine the vials for evidence of damage. DO NOT USE any vials that exhibit any cracks or defects. Discard any defective vials. On rare occasions, the glass neck may be cracked and the neck may break during removal of the flip-off cap or in handing. Also, on rare occasions a vial may not be sealed sufficiently. In either case, the contents of the vial may leak or spill. If the vial has been inoculated, treat the leak or spill with caution, as pathogenic organisms/agents may be present. Before discarding, sterilize all inoculated vials by autoclaving.

To minimise the risk of leakage during inoculation of specimen into culture vials, use syringes with permanently attached needles or securely fastened Luer-LOK® brand tips.

Comprehensive reviews of laboratory safety procedures may be found in recognised publications of the Centres for Disease Control and the American Society for Microbiology.

(ii) History of disease:

In 1882, Robert Koch announced that tuberculosis was caused by Mycobacterium tuberculosis, the tubercle bacilli. This discovery ultimately led to ways of decreasing transmission and effective treatment modalities. Prior to Koch’s discovery, TB was believed to be constitutional, a form of tumour of abnormal gland (Bloom & Murry, 1992). In the 1887s and 1880s, scientists generally held that TB resulted from hereditary predisposition, juxtaposed with a bad environment and /or improper living (Bates, 1992).
Robert Koch (1882) isolated mycobacterium tuberculosis and proved its causative role in tuberculosis. On August 18th 1881 the first animal's two guinea pigs were vaccinated with tuberculosis material. On March 24th 1882 seven months after the experiments has begin Robert Koch reported in his lecture 'about tuberculosis' to the Berlin physiological socially on the results of his experiments. This lecture contains in its basic concepts all that are know ever today about the bacteriology of this disease.

A hundred year later, we today still benefit from the ideas, experiments (Lock 1983).

The generic name mycobacterium (Lehmann and Neumann 1896) was given to a group of bacteria that grew as mould like pellicles formed by M. tuberculosis on liquid media.

The only genus mycobacterium in the family mycobacteriaceae are usually straight or slightly carved rods shaped organism with parallel sides and rounded end, usually 1-4 um x 0.3 – 0.6 um in size which frequently from small clumps Cells of M tuberculosis are often arranged in serpentine cords (plate-10) (Goren et. al. 1971). The morphology varies from species to species of cells of M. xenopi are often filamentous with occasional branching and areola hyphase, those of M. Kansasii are after elongated with a distinct banded or beaded appearance, while those of M. avium are often almost coccoid (Runyon, et.al. 1974). Some of the saprophytic species occasionally cause diseases in animals and mans. These have a number of unsatisfactory epithets including "atypical; opportunistic" Tuberculoid and MOTT (Mycobacterial other than typical tuberele) bacilli. The more aesthetic term "nyrocine mycobacteria; proposed by Grange and Collins (1983) has not
unfortunately, entered general usage; perhaps the best name at present is environmental mycobacteria).

The genus mycobacteria is very well classified as a result of extensive studies under taken by the international working group on mycobacterial taxonomy (Wayne, and Dietz. 1971; Wayne et.al.1981; Kubica et.al. 1972; Meissner, and Schroder. 1974; Saitro and Gordon. 1977). The genus is distinguished by characteristic antigenic patterns (Stanford and Grange. 1974) and mycolin acid structure G+C content of DNA 66-72 mol% except mycobacterium leprae has 55 mol%. Except mycobacterium leprae has 55 mol%.

Mycobacteria can potentially be recovered form a variety of clinical specimens, including upper respiratory tract collections (i.e. sputum, bronchial wash, bronchoalveolar Lavage, bronchial biopsies) laryngeal swab, Nasopharyngeal swab Body fluid i.e. Plural fluid, pericardial fluid, joint aspirate, Gastric Aspirate, Peritoneal fluid C.S.F. stool, urine, pus, tissue biopsies and deep needle aspirations of virtually any tissue or organ (Krasnow I, Wayne L.G. 1969).

Tuberculosis (TB) is an ancient disease. Physical evidence from Egyptian mummies and Stone Age skeletons suggests that TB has existed in humans since antiquity (Bloom and Murry. 1992 ; Joklik, et al.1992; Zimmermans,1979). TB was epidemic in Europe in the 18th and 19th centuries when vast numbers of people died from it. TB has been variously called, "consumption," "phthisis," "the white death," "the white plague," and "captain of all these men of death." Until the 20th century, TB was the most important cause of death among infectious disease worldwide (Bloom and Murry. 1992; Keers. 1978; Meyer. 1991). Even today TB remains the leading cause of death among infectious disease Worldwide (Bloom
Koch’s discovery of the cause of TB, unfortunately, did not result in its cure, although his premature announcement in 1890 that he had discovered a substance to prevent the development of tubercle bacilli in humans created a sensation (Leibowitz, 1993). Even before Koch’s discovery, the belief that sunlight, healthful food, and fresh air could fight tuberculosis took hold (Ernest & Sbarbaro, 1993). Hermann Brehmer established the first sanatorium in 1884 in Europe worth the theory that altitude and exercise could cure TB (Bloom and Murray 1992). In the belief that the aroma of spruce or pine trees was therapeutic, sanatoriums were established in mountainous areas, particularly in New York State (e.g. The ray brook State Tuberculosis Hospital, the Surmount Veterans Administration Hospital, and the Will Rogers Sanatorium), Colorado, and North Carolina (especially near Asheville) (Meyer, 1991). Unprecedented health campaigns (e.g., the Easter Seal campaign) against TB swept the nation in the first half of the 20th century.

Bates (1992) argues that the building of institutions for treatment of consumptives sprang from three main goals: (1) an institution could “correct the moral decay of an urban society and save the souls of the helplessly ill” (p.330); (2) segregation could prevent the spreads of disease to others; and (3) treatment in an institution could effect cure, thus returning a patient to a productive life. A fourth goal of institutions, never fully realise, was linking care to eradication of TB through research. During the era of institutionalisation of TB patients, home care was largely unsuccessful as a strategy to treat and prevent TB (Bates, 1992). During the period, however, African-Americans were largely cared for in the home
since institutional care was either unavailable to them or, if available, was substandard (Bates, 1992).

In the decades before the introduction of chemotherapy, the armamentaria in the battle against TB had been highly unpredictable in bringing about a cure. In addition to segregation of TB patients (and sometimes their families), chaulmoogra oil (a tree derivative once used to treat leprosy) and various chemicals, dye, metal, salts, and antibiotics (e.g., sulfonamides and penicillin) were tried. Surgical intervention, called “collapse therapy” (i.e., therapeutic or artificial pneumothorax), involved the injection of air into the pleural space to effect lung “collapse,” thus allowing the infected lung to rest. If this intervention was unsuccessful, thoracoplasty or phrenic crush might also be tried to decrease the lung’s mobility and/or effect cavity closure. Although these procedures were not without complications (e.g., infection, hemoptysis, and sudden death) some patients improved or recovered from their disease (Bates, 1992; Meyer, 1991). Thoracoplasty was the most successful of the surgical interventions prior to the introduction of chemotherapy and was reported to have returned thousands of patients to a normal life (Meyer, 1991).

Treatment of TB prior to the introduction of chemotherapy was largely aimed at its early diagnosis. By the late 1930s diagnosis could be established in those believed to be infected through tuberculin skin tests and radiographs (Bates, 1992). Once diagnosed, the patient’s family members could be examined and the patient could be offered treatment, often in a sanatorium. Those with the means could travel to better climates for treatment in private sanatoriums. From 1900 to 1940, care of TB patient gradually shifted from homes to institutions, largely
government supported. During this period, few African Americans entered sanatoriums even the public ones, because the facilities were segregated. Because of the Depression, however most private sanatoriums had disappeared by the 1930s (Bates, 1992).

Following the introduction of chemotherapy, the care of TB patients began to shift from sanatoriums to general hospitals, hastened by a recommendation by the American College of Chest Physicians (1972). By 1993, only 17 states had TB sanatoriums, and by 1981 most of these states had also begun to use general hospitals for TB treatment (Reider, 1989). The sanatoriums had become largely obsolete with the advent of short hospitalisations and ambulatory treatment. Over time, most of these sanatoriums were demolished or converted to other uses.

The discovery of drugs to treat TB effectively held the potential for a cure and marked dramatic changes in care delivery. Streptomycin was discovered in 1993 by Selman Waksman (Earnest & Sbarbaro, 1993), followed by 11 more drugs by 1967, although only two, isoniazide and rifampin, were considered first-line drugs. Drug treatment allowed for most persons with active TB to be cured within a few months and to return to normal lives within a few weeks after chemotherapy began. Thus, the major challenges in TB treatment then, as today, included prevention of intervention activities, early identification of infected individuals, and implementation of interventions to ensure their adherence to treatment.

Although the HIV/AIDS epidemic has more recently highlighted the damaging elements of fear on society, fear also played an important role in the battle against TB in the early 20th century during the anti-TB campaigns. Growing concerns about TB in the 20th century fed xenophobia and a nativism movement.
resulting in restrictive immigration laws, possibly aimed at the “germs” of socialism and anarchism (Rothman, 1992). The association of TB and poverty also led to the labeling of persons with TB as “one lungers.” Fear of TB during the era even had a name: phthisiophobia (Rothman, 1992).

(iii) Pathogenesis of the disease:

It is important to understand the difference between tuberculous infection and tuberculosis. Tuberculous infection may be present with or without disease (tuberculosis). Persons who have living tubercle bacilli present without clinically active disease are considered to have tuberculous infection. Such individuals usually have a positive tuberculin skin test but do not have symptoms of tuberculosis and have negative bacteriological studies. They are not infectious to others. Those individuals with tuberculous infection are at risk of contracting clinical disease, particularly if their immune system becomes impaired. Such a conversion may not who are not only infected with *M. tuberculosis*, usually having a positive tuberculin skin test but who have clinically active disease usually with symptoms (although some time these are non-specific) and who are infectious to others (American Thoracic society, 1992; Centre for Disease Control, 1990).

The pathogenesis of tuberculosis occurs in two phases (1) the initial (primary) infection with tubercle bacilli and (2) the subsequent development of tuberculosis (Hopewell, 1988). The pathogenesis of tuberculosis depends on a number of factors such as the host’s innate body defenses, nutritional status, age and possibly, the dose of bacteria implanted in the lungs (inoculum effect).

The droplet nuclei that are inhaled and are small enough to reach and implant on the alveolar surface usually contain only a few tubercle bacilli. If
this is the person’s first encounter with TB, alveolar macrophages that normally scavenge the alveolar surface ingest the tubercle bacilli and can kill or remove them. This nonimmunologic response can be effective in preventing infection if relatively few bacilli are implanted on the alveolar surface. Other factors influencing outcome include the virulence of the tubercle bacilli and characteristics of the alveolar macrophage such as their microbicidal capacity. If large numbers of bacilli are present, the microbicidal capacity of the non-nonsensitize macrophages may be exceeded, allowing the surviving organisms to multiply and establish a tuberculous infection (Hopewell, 1988; Nardell, 1993). If alveolar macrophages kill the tubercle bacilli at the time of the first exposure, then infection dose not become established, and the tuberculin skin test remains negative (Pitchenik & Fertel, 1992).

Early after \emph{M. tuberculosis} infection, an inflammatory response occurs within the lung. When this localized inflammation heals, a calcified parenchymal lesion called the primary or Ghon complex is left. Bacilli are still proliferating because cell mediated immunity has not developed (Dunlap & Briles, 1993). \emph{M. tuberculosis} can replete within the macrophages causing lysis, the escape of the tuberculosis bacilli and the infection of other alveolar macrophages (Edwards & Kirkpatrick, 1986; Moulding, 1983; Pitchenik and Fertel, 1992).

At the time of the initial tuberculous infection, tubercle bacilli are transported within macrophages to regional lymph nodes in the hilum of the lung, ultimately allowing their entry into the bloodstream where the tubercle bacilli can then be seeded throughout the body, creating potential sites at which future extrapulmonary disease may develop. This lymphohematogenous dissemination of
tubercle bacilli is usually asymptomatic; it occurs before the person acquires tuberculin hypersensitivity (Joklik et al., 1992). Within the bloodstream, circulating tubercle bacilli are cleared by reticuloendothelial organs. However, bacterial multiplication continues in the lung apices where oxygen tension is high and the environment is favorable and in such areas as the epiphyses (growing ends) of the long bones, all of which have favorable concentrations of oxygen (Joklik et al., 1992). Most of these lesions remain asymptomatic and heal with the development of cell-mediated immunity and delayed hypersensitivity that occur within a period of 3 to 8 weeks after infection, during which time the tuberculin skin test becomes reactive unless anergy is present (Dunlap & Briles, 1993; Pitchenik and Fertel, 1992).

At the time that the initial infection occurs, the immunologic defenses are stimulated but require 3 to 8 weeks to become effective (Joklik et al.; 1992; Pitchenik and Fertel, 1992). When the inhaled tubercle bacillus is phagocytyized by the alveolar macrophage, mycobacterial antigens are released through lysis of the organisms. These antigens are processed by the macrophage and presented to the T-lymphokines. The lymphokines include macrophage-activating factor, chemotactic factor, migration inhibition factor, and mitogenic factor. The release of these mediators causes proliferation of lymphocytes and infiltration of the affected area with blood lymphocytes and infiltration of the affected area with blood lymphocytes as well as monocytes and activated macrophages are much more effective at killing tubercle bacilli than the nonactivated macrophages that responded earlier to the initial infection (Edwards & Kirkpatrick, 1986; Hopewell, 1988; Moulding, 1994; Reynolds, 1987).
This acquired cell-mediated immunity constitutes the major determinants of the host resistance to the further development of disease (Hopewell 1988; Murray and Mills, 1990). The immunologic response kills bacteria and contains any remaining organisms by walling them off in a granulomatous tissue reaction (Daniel & Ellner, 1993; Joklik et al., 1992; Murray 1989, Murray and Mills, 1990). The sequestered bacilli remain viable but become dormant, allowing the infection to remain latent as long as cell mediated immunity remains intact. This tubercle formation or hypersensitivity granulomas is composed of macrobacterial antigens becoming enlarged, and often referred to as epitheliod cells. Often a peripheral collar of fibroblasts, macrophages, and lymphocytes surrounds it. Some times caseous necrosis occurs in the central part, and a “soft” tubercle results. These lesions may calcify, resulting in primary or Ghoncomplex (Daniel & Ellner, 1993; Dunlap & Briles, 1993; Joklik et al., 1992; Kuritzkes & Simon, 1991). Should calcification of a hilar lymph node occur, this lesions and the Ghon complex collectively make up the rank complex (Edwards & Krikpatrik, 1986; Hopewell, 1988; Moulding 1983).

Of great clinical significance is whether the caseation necrosis remains a cheeselike consistancy or whether it undergoes softening and liquefaction (Dannenberg, 1994). In the majority of people who are infected after the tubercle is formed, the person is asymptomatic and infection is latent as the tubercle bacilli are dormant and contained by the immune system, and the tuberculin skin test will be positive. This is true in about 90% of exposed individuals. In some persons, however, cell-mediated immunity does not completely control primary tuberculosis, and liquefaction necrosis occurs with the primary infection evolving
into clinical disease (Nardell, 1993). The mechanisms that cause liquefaction are unclear but when it occurs there is a massive increase in the number of tubercle bacilli. As liquefaction of a caseous mass occurs in the lung tissue, the liquified material is expelled via the bronchi and a cavity forms. The number of bacilli in the resulting cavity will be very high probably because of the presence of softened caseum and a high concentration of oxygen. Both of which are conductive to the growth of the organism (Joklik et al., 1992). Tuberculous pneumonitis may result, and patients with cavity tuberculosis typically exhibit coughing and systemic symptoms such as fever, anorexia, and weight loss (Nardell, 1993). The consequences of this large population of bacilli existing in a cavity include the possibility of bronchogenic spread of bacilli to other portions of the lung and increased infectiousness of the infected person.

As previously stated, the adequacy of the immune response will determine whether tuberculous infection proceeds to disease. A relative minority of infected persons actually develop tuberculosis disease. Approximately 90% of infected individuals will remain free of clinical tuberculosis during their lifetime (Dunlap and Briles, 1993; Nardell, 1993). Of the remaining 10% of infected individuals, half of the total number of infected individuals (5%) will develop disease within the first few years after infection (early), where as the remaining half will develop disease following a much longer interval (late), in some cases as long as several decades after the initial infection (Dunlop and Briles, 1993; Kuritzkes and Simon, 1991; Murray, 1989 ; Murray et al., 1980). Thus, in most cases the outcome for primary infection is control and resolution although viable bacilli can remain in the granulomas (and in other body sites ) allowing reactivation if cell-mediated
immunity is compromised. Typically, the only signs of resolved primary tuberculous infection are reactivity to the tuberculin skin test and sometimes chest radiographic evidence of the Ghon or Ranke complex (MacGregor, 1993).

The proper functioning of the elements involved in the immune response to *M. tuberculosis* as detailed above prevents progression to tuberculosis disease in the infected host and also boosts the immune response to any subsequent exposure to tubercle bacilli, thus preventing implantation and development of new infection. Any impairment of cell mediated immunity creating an inadequate immune response therefore, have the following possible consequences:

(i) endogenous reactivation - reactivation of previously acquired tuberculous infection; or (ii) exogenous infection or reinfection - acquisition of new infection with tubercle bacilli from an external source (Murray and Mills, 1990; Nardell, 1993).

Reactivation of a previously acquired tuberculous infection leading to clinical disease is most often seen after 50 years of age and is most common in males. This reactivation can result from a breakdown in cell-mediated immunity in the host from such causes as malnutrition, drug or alcohol use, diabetes, HIV-infection, other causes, and perhaps even severe stress (Sherris, 1990). These factors modify the immune response through their effects on the cells that initiate and modulate the immune response. In addition to the roles of other cell types in the acquisition of cell-mediated immunity, discussed earlier, subsets of the T-lymphocytes, the helper and suppressor cells, serve as regulators whose presence and activity determine the adequacy of the immune response (Moulding, 1983).
The most common site for reactivation is in the apex of the lung where oxygen tension is high.

(iv) Antigenic structure:

The mycobacterial antigens may be broadly classified as (1) cytoplasmic (soluble) or cell wall lipid-bound (insoluble), (2) according to their chemical structure (carbohydrate or protein) or (3) by their distribution within the genus. Antigens have been extensively used to classify, identify and type the mycobacteria (Fig-1).

![Antigenic sharing between mycobacteria and related genera](image)

**Fig.1 : Antigenic sharing between mycobacteria and related genera (species-specific, or group iv, antigens are not shown)**

Soluble antigens Up to 15 lines of precipitation are demonstrable when ultrasonicates of mycobacteria are tested against homologous antiserum by
immunoelectrophoresis (Daniel and Janicki 1978) or double diffusion in agar gel (Stanford and Grange 1974) while up to 90 antigens are demonstrable by the more sensitive technique of crossed immunoelectrophoresis (Closs 1980). Soluble antigens are divisible into four major groups (Fig-1) (Stanford and Grange 1974): those common to all mycobacteria (group-i); those occurring in slowly growing species (group-ii) and those occurring in rapidly growing species (group-iii), and those unique to each individual species (group-iv). This antigenic distribution suggests that the mycobacteria evolved from a common ancestral from and that there was an early division of the genus in to the slow and rapid growers. Some of the group iii antigens are also found in the genus Nocardia (Stanford and Wong 1974). Many of the common (group-i) antigens are also found in the nocardiae and some are detectable in related genera such as Corynebacterium and Listeria. This intergeneric sharing of antigens is probably responsible for the notorious lack of specificity of serological tests for tuberculosis.

Immunoelectrophoretic analysis of culture filtrates of M. tuberculosis (Daniel and Jannicki 1978) revealed 11 arcs of precipitation, which were numbered for reference purposes. Antigens 1, 2, and 3 are, respectively, arabinomannans, arabinogalactans and glucans that are distributed throughout the genus; antigens 6, 7 and 8 are widely distributed proteins while antigens 5 is a proteins of mol. wt 28 500 - 35 000 and is specific for M. tuberculosis. Closs (1980) proposed a more elaborate reference system based on 30 numbered antigens of BCG demonstrable by two-dimensional polyacrylamide- gel electrophoresis. Several of these antigens have been further characterised (Harboe et. al. 1986) and some have been shown to be enzymes (Harboe and Wiker 1986).
Tuberculin. The earliest preparations of mycobacterial antigens used experimentally was Koch’s tuberculin. The first of these was Old Tuberculin, a filtrate of old broth cultures of tubercle bacilli concentrated by evaporation (Koch, 1891). Later he (Koch, 1901) prepared New Tuberculin by finely grinding vacuum-dried tubercle bacilli in a mortar and reconstituting in a glycerol-water mixture. In early epidemiological studies with tuberculin, pioneered by Clemens Von Pirquet, non-specific reaction due to constituents of the medium were frequent. Accordingly, Seibert and her colleagues attempted to separate mycobacterial antigens from impurities in heat-concentrated old culture filtrates by precipitation of proteins by 50% ammonium sulphate. The resulting preparation, Purified Protein Derivative (PPD) (Seibert and Glenn 1941), was and still is used extensively. Even this preparation was far from ideal because the processes of autolysis heating and protein precipitation cause considerable denaturation of the antigens particularly the species-specific ones (Stanford and Rook 1983). Many attempts were therefore made to isolate specific antigens by means of all available proteins-fractionation techniques reviewed by (Daniel and Janicki, 1978), but no reagent suitable for clinical use was developed. To avoid denaturation during preparation, Stanford and his colleagues produced range of New Tuberculin by means harvesting mycobacteria from non-antigenic Sauton’s medium, washing them, disrupting them in an ultrasonicator, separating cytoplasm from cell-wall debris by centrifugation, sterilising the supernate by repeated membrane filtration and standardising the proteins contents of the filtrate. The first of these reagents was Burulin, used for studies on M. ulcerans infection (Stanford and Revill.
1975) Subsequently new tuberculin were prepared from *M. tuberculosis* and a
range of environmental mycobacteria (Stanford and Rook 1983).

Insoluble (agglutination) antigens – Mycobacteria that from stable smooth
suspension may be identified or typed by agglutination tests. The agglutination of
the *M. avium-intracellulare* group has been studied extensively by Schaefer and
his colleagues. Serotypes are identifiable in several other species but not,
unfortunately, in *M. tuberculosis* which is rough and readily autoagglutinates. The
responsible antigens have been identified as the sugar moieties on mycosides these
include the major antigenic phenolic glycolipid of *M. lepera*.

Mycobacterial chromosomes were found to have mol. Wts of (2.5-5.55) x
10, with those of the major pathogens *M. tuberculosis* and *M.leprae* being at the
lower end of the range (Bradley 1972, Baess and Mansa 1978).

For comparison the mol wt of the genome of *E. coli* is 2.5x109. In addition
to the main chromosome, some strains contain one or more plasmids (Crawford, et
al. 1981); there is some evidence that, in *M. avium*, these affect colonial
morphology, resistance to antimicrobial agents and virulence (Mizuguchi 1981).
The G+C contents of the mycobacteria is high, 66.71 mol% (Baess and Mansa
1978), although in *M. leprae* it is only 55 mol% (Imaeda, et al.1982) DNA pairing
(Baess and Bentzon 1978) confirms the species boundaries as determined by other
methods and also reveals a low homology between slowly growing and rapidly
growing strains, supporting the serological evidence that these are evolutionary
divergent subgenera. Similar technology has also been used to produce
‘DNA probes’ both for detecting mycobacterial genomes in sputum (Pao and Lin.
1988) and for identifying strains (McFadden et. al. 1987a)
(v) Colony morphology:

Information obtained from observation of colony morphology often proves helpful in the identification of culture organism for the best observation of colony morphology, the microbial suspension inoculated on to midia after diluted sufficiently to yield isolated colonies. The following terms used to describe mycobacterial colonies on culture media.

**Surface:** This may be smooth, rough or cored "X"colonies. The latter Ardennes, compact, smooth-to rough, slow growing colonies that often exhibit tiny stick-like projections at the periphery when viewed by transmitted light. Because the colonies are almost unique to *M.xenopi*, they are referred to as "X"colonies (the latter coming form the species name).

**Elevation and colony from:** Descriptive terms included flat, convex, and pyramidal, umbonate (raised center), rosette (lobate), and doughnut.

**Edge:** This may be entire, irregular, flat and spreading. Pigment (in light and dark): Recorded this as nonpigmented (buff), pigmented (name the colour, e.g. yellow, orange), or "Greening" (plate-7 B) (some culture absorb the malachite green dye from L-J slants during the incubation, or when they are stored in the refrigerator).

Two additional terms commonly used in the mycobacteriology laboratory to refer to culture growth are “eugonic” and “dysgonic”. The term “eugonic describes culture growth that is luxuriant (i.e., large easily seen colony), whereas “dysgonic” refers to tiny, difficult to see colonies that might be said to be grown poorly.
M. fortuitum - The colonies are soft, butyrous, hemisphere and multilobate or rough with heaped centres although nonpigmented they appear green going to absorption of malachite green (plate-7 B).

M. Kansaii - The colonies are smooth or rough; although nonpigmented when grown in the dark they become lemon yellow when exposed to light for 1 hour; on continuous light exposure the characteristic orange-red crystals of beta carotene appear (plate-9).

M. Szulgai - The colonies smooth to rough pyramid-shaped with somewhat irregular periphery scotochromogenic (orange) when incubated at 37 °C and photochromogenic at 25 °C continuous light exposure may result in formation of red crystals (Plate-9).

M. avium-intracellulare - The colonies are smooth dome shaped and buff coloured; rough, wrinkled colonies are sometimes seen; cultures sometimes appear impure, since more than one type colony may appear on the same culture.

Mycobacterium avium-intracellulare-scrofulaceum: The three species that make up this complex, M. avium, M. intracellulare and M. scrofulaceum, are difficult to differentiate except by technique usually available only in reference laboratories colony on egg media at 14-21 days are about 1 mm in diameter, smooth, white and domed but growth may be effuse. Some strains produce a feeble yellow pigmented and those identifiable as M. scrofulaceum a deeper yellow pigment, unaffected by light; hence they are sometimes referred to as the scrofula Scotochromogen. The bacilli are small, about 1.0 X 0.5 um often-appearing almost coccoid.
Mycobacterium chelonel-The organism grows rapidly (two to three days) when subculture on nutrient agar and egg medium and produces no pigment. The colonies are white, moist, soft, and domed, 2-3 mm in diameter.

Mycobacterium fortuitum-The colony on nutrient agar and egg media at three days is 2-3 mm in diameter, white or buff and either rough or smooth and domed. The bacilli show no distinctive morphology, being about 2-3 x 0.5 um, with some fatter forms. They stain solidly (plate-11A).

(vi) Morphology of organism:

The mycobacteria are usually straight curved rod shaped organism with parallel sides and rounded end hardly 1-10 μm long and 0.2 –0.6 μm wide in size which frequently form small clumps (plate-5A). Cells of M. tuberculosis are often filamentous with occasionally branching and aerial hyphen. those of M. kansasii are often elongated with a distinct banded or beaded appearance while those of M. avium are after almost coccoid (Runyou 1974).

Smear made from a positive vial (GI = 100 or more) may give an indication of the type of mycobacteria growing in the medium. Presence of serpentine cords and clumps (plate-10) is a characteristic of M. tuberculosis (M bovis and M.kansasii may also form cords). This characteristic is more pronounced in 12 B medium than on solid media. Other mycobacteria also have some characteristic morphology, such as M.avium, M. intracellulare that show branching with long strands of bacilli. Although sometimes smear morphology may help in distinguishing TB from MOTT bacilli, confirmation is necessary.

M. tuberculosis and M. bovis grow more slowly than any other mycobacteria. This characteristic may be helpful in tentatively differentiating
between TB and other mycobacteria. This difference is even more distinct in subcultures. In primary cultures, several factors, such as the number of organisms present in the specimen, decontamination of the specimen, etc., influence the growth rate of mycobacteria to some extent. However, on subculture, mycobacteria are uninhibited and thus reveal a greater difference between the growth rate of TB and MOTT bacilli (Siddiqi 1985a).

*M. tuberculosis* and *M. bovis* exhibit the slowest growth and the daily GI increase is usually 2 to 3 fold while other mycobacteria may show a daily increase as high as 10 fold. *M. Kansasi* is one of the slowest growing MOTT bacilli in 12B medium, while *M. avium*, *M. intracellulare* and *M. simiae* grow very rapidly. The average detection time of TB during primary culture is usually longer than the average detection time of MOTT bacilli.

Visually, *M. tuberculosis* growth in 12 B medium display small specks or clumps with a clear medium. Other mycobacteria grow in very small particles and may give slight turbidity to the medium.

Thus, the daily GI increase provides some assistance in the differentiation of TB from other mycobacteria. With some experience, one can tentatively differentiate the two major categories of mycobacteria by observing the growth rate and appearance of growth in 12B medium although further confirmation is necessary before reporting results.

(vii) History of BACTEC

All types of clinical specimens, pulmonary as well as extra pulmonary can be processed for BACTEC primary isolation in a manner similar to conventional isolation procedures. Details are provided in the CDC Manual “Procedures for the

Sputum specimens must be digested (liquefaction) and decontaminated (selected elimination of bacteria other than mycobacteria). Other specimens, which may contain organic material and contaminating bacteria, should be liquefied and decontaminated in the same way as sputum. Specimens which contain little organic matter and are collected aseptically do not require the liquefaction, decontamination procedure. Clinical specimens collected in large volume require a centrifugation step to concentrate the mycobacteria present in the specimen.

BACTEC 12B is a liquid medium, which does not allow observation of colonial morphology. However the GI in 12B medium can be characteristic of certain types of Mycobacterial growth.

With conventional techniques, once a culture is positive, definitive identification is carried out by performing a battery of biochemical test using growth obtained the primary isolation medium, or on a subculture if the original growth is not sufficient. The time required for speciation is usually 3 to 6 weeks, and may be longer when subculturing is necessary. Clinically, as well as epidemiologically, early differentiation of M. tuberculosis (TB) from mycobacteria other than M. tuberculosis (MOTT) is more important than a complete speciation of the isolated culture. Therefore, the BACTEC approach has been directed towards a rapid and reliable differentiation of TB from other mycobacteria.

The BACTEC procedure for drug susceptibility testing of mycobacteria is based on the same basic principle employed in the conventional method. The only
difference is that a liquid medium is used and instead of counting colonies after about 3 weeks, the growth is monitored radiometrically and the results are reportable within 4 to 5 days. BACTEC drug susceptibility is determined by following the modified version of the conventional proportion method. The critical proportion for resistance is taken as 1% for all antituberculosis drugs. This means that if 1% or more of the test mycobacterial population is resistant, the culture is considered resistant for laboratory reporting purposes. Resistance is determined by comparing the rate of growth in the control and the 12B vials containing test drug.

Several published studies have reported that results obtained by the BACTEC method compared well with the conventional proportion method (employing 7H10/7H11 media) or the resistance ratio method (employing L-J egg medium (Bannister, 1985, Roberts et al 1983, Siddiqi et al 1981; Siddiqi 1985; Steadham 1985; Vineke et al 1982). The accuracy and reproducibility of the BACTEC method has also been evaluated, with excellent results (Good. 1984; Siddiqi. 1984; Terrand 1985; Hawkins et al 1988).

Initially, it was recommended that the BACTEC drug susceptibility test should be read daily including the weekend. However, later studies indicate that a non-weekend protocol can also be adopted successfully (Hawkins 1986).

Direct drug susceptibility testing of AFB smear-positive specimens can be successfully carried out using the BACTEC method. In the first studies, reportable susceptibility results were obtained from more than 67% of the AFB smear-positive and more than 86% of the culture positive specimens with an average reporting time of 10.7 days for *M. tuberculosis* (Broman, 1982). There was
excellent agreement when the BACTEC direct susceptibility test results were compared with results obtained by the conventional plate method.

At the time the first direct susceptibility study was carried out, there was no BACTEC test available for the identification of mycobacteria. With the introduction of the BACTEC NAP TB differentiation test, a new approach has been adopted. Now, complete information about isolation and identification (TB or not TB) is available by the time direct susceptibility results are reported. Field trials of the direct susceptibility test together with identification indicated good agreement with the conventional results. This allows significantly earlier reporting (Libonati, 1984; Stager, 1984).

The BACTEC drug susceptibility testing procedure is well established for *M. tuberculosis*. However, if an appropriate inoculum is used, susceptibility testing of MOTT bacilli can also be performed with reproducible results. Good correlation of BACTEC and conventional drug (S.I.R.F->) susceptibility test results has been reported for *M. Kansasii* (except streptomycin) and *M. marinum* (Hawkins, 1984). On the other hand, results for some other MOTT bacilli do not correlate as well as those for *M. tuberculosis* (Canetti, 1969; Steadham, 1985). In general, in 12B medium, MOTT bacilli show greater susceptibility to streptomycin, rifampin and ethambutol, and are almost always resistant to INH. It is possible that more drug-cell contact in the liquid medium and a more rapid result (4 days as compared to 2-3 weeks) may influence the overall susceptibility test results.

The difference in the susceptibility-testing pattern of MOTT bacilli provided by BACTEC and conventional methods is of great interest and deserves further investigation. At present, there is no accepted method for susceptibility testing of
nontuberculosis mycobacteria. Susceptibility testing is generally carried out following the conventional methods recommended for *M. tuberculosis*. However, susceptibility testing of MOTT bacilli has been reported by several investigators with known antituberculosis drugs as well as newer antimicrobial (Steadham, 1985; Heifets, 1988; Heifets, 1991; Lee and Heifets 1987). A recent multi-center study established a standardized procedure to carry out susceptibility testing of *M. avium* with the radiometric method and reported good inter-laboratory reproducibility (Siddiqi, 1993).

Bacteriological investigation plays a key role in the diagnosis of tuberculosis. Demonstration of acid-fast bacteria (AFB) in smears (plate-5A) made from a clinical specimen provides preliminary diagnostic information while the isolation of AFB on culture media provides essential information on the properties of mycobacteria seen on the smear.

Egg-based media, such as Lowenstein Jensen (L.J), have been used to cultivate mycobacteria. In 1958, Middlebrook and Cohn devised an agar-base medium to permit more rapid detection of mycobacterial growth. However, it still required an average of three weeks to recover mycobacteria from clinical specimens.

Once mycobacteria are recovered from a specimen, it is necessary to identify these AFB because of the prevalence of mycobacteria other than tuberculosis (MOTT bacilli or atypical or non-tuberculous mycobacterial). It is extremely important to differentiate *Mycobacterium tuberculosis* (TB) from other mycobacteria because of its communicability. An early differentiation helps the physician to manage the patient and potential contract appropriately. Thus,
considerable cost and labour may save if definite information is available early (Alexander, et. al. 1983). In laboratory practice a variety of biochemical tests, especially the niacin test, are used to identify mycobacteria.

In order to treat the patient effectively, the clinician needs information about the drug susceptibility of the isolated culture. Susceptibility testing with Middlebrook 7H110 agar-base medium has overcome the limitation of egg-based media that require inspissation, a step which can affect the potency of many drugs added to the medium. Moreover, it was established that the use of 2-10% CO₂ helped the growth of mycobacteria on 7H110 medium and addition of casein hydrolysate encouraged the growth of many drug resistant strains of M.tuberculosis. (Cohn et. al. 1968, McClatchy et. al. 1976).

In 1969, Deland and Wagner developed a technique for automated detection of the metabolism of bacteria by measuring the ¹⁴CO₂ liberated during the decarboxylation of ¹⁴C-labelled substrates present in the medium. This technique has been applied successfully to blood culturing, detection of antibiotic effect on the bacterial growth, Neisseria sp. differentiation by substrates metabolism, and serum assay of aminoglycoside antibiotics. Cummings and co-workers in 1975 carried out preliminary work that showed the same principle could be applied to detect growth of M.tuberculosis (Cummings et.al. 1975). Middlebrook further developed the technique and introduced 7H112 liquid medium containing a ¹⁴ C-labeled substrate specific for mycobacterial growth (Middlebrook et. al. 1977). He reported a significant time saving in the primary isolation of mycobacteria from clinical specimens using the new radiometric medium.
(viii) Principal of BACTEC:

The BACTEC TB medium (12B) is an enriched Middlebrook 7H9 broth base. Mycobacteria utilise a $^{14}$C labelled substrate (fatty acid) present in the medium and release $^{14}$CO$_2$ into the atmosphere above the medium. When the vials are tested on the BACTEC460TB System (plate-2) instrument, the gas is aspirated from the vial and the $^{14}$CO$_2$ radioactivity is determined quantitatively by increasing reading in an ion chamber in terms of numbers on a scale from 0 to 999. These numbers are designated as the Growth Index (GI). The GI numbers are displayed by the BACTEC 460TB system instrument and are also printed along with the identifying rack and bottle numbers (100GI units are approximately equal to 0.025uCi). The daily increase in the GI is directly proportional to the rate and amount of growth in the medium. Growth is detected by increasing reading in an ion chamber.

The BACTEC instrument also introduces fresh 5-10% CO$_2$ in air into the medium headspace every time a vial is tested. This enhances the growth of mycobacteria. The instrument automatically tests 60 vials at the rate of approximately one vial every 82 seconds and stops at the end of the run.

If an inhibitory agent is introduced into the medium, inhibition of metabolism is indicated by reduced production of $^{14}$CO$_2$ when compared to a control having no inhibitory agent. This basic principle is applied for drug susceptibility testing and in differentiating TB from other mycobacteria.

The BACTEC 460 TB System instrument must be used with a special TB hood when employed for mycobacteriology. The TB hood provides HEPA filtered exhaust air and negative pressure in the test area. In addition, the TB hood is
equipped with an ultraviolet light source in the test area. The unit is designed for automatic testing of vials and must not be used for inoculation or subculturing in place of a biological cabinet.

(ix) Development of BACTEC:

The development of 7H12 medium (BACTEC 12A) led to several studies that reported excellent recovery of mycobacteria from sputum as well as extrapulmonary specimens (Bannister 1985, Dameto et al. 1983). This study used an inoculum volume of 0.1ml per 2.0ml of medium (12A). Subsequent studies (Libonati, 1983; Morgan, 1983) showed that improved recovery occurred if 4.0ml of medium per vial were inoculated with 0.5 ml of inoculum. A modified 7H12 was then introduced and designated as BACTEC 12B medium.

Since the BACTEC system utilises a liquid medium. It is important to add an antimicrobial supplement to suppress growth of contaminating micro-organisms that may survive the decontamination process. Initially a modification of Mitchison's antimicrobial mixture which contained polymyxin B, amphotericin B, carbenicillin and trimethoprim (PACT) was added to 12A medium (McClatchy et al. 1976, Middlebrook et al. 1977). With the increased inoculum size in 12B medium, it was found that the contamination rate was higher (Morgan, 1983, Libonati, 1983). Siddiqi et al reported another antimicrobial mixture (PANTA) which suppressed contamination significantly better than PACT (Siddiqi and Hwangbo, 1986). PANTA contains polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin. Several studies indicated superior results with PANTA (Libonati, 1986, Stager 1986, Tice and Roberts, 1986).
Although BACTEC TB medium supports rapid growth of most mycobacteria, occasional strains of *M. tuberculosis*, such as isolates from treated chronic cases, may grow poorly. Recently, a growth promoting substance, polyoxyethylene stearate (POES), has been reported by Siddiqi, 1985. When added to BACTEC TB medium, this substance enhanced growth of those strains, which grew slowly or poorly. This growth promoting substance has been incorporated in the PANTA Reconstituting Fluid (RF), which should be used to reconstitute lyophilized PANTA Supplement.

Employing the same basic radiometric principle, drug susceptibility testing for streptomycin, isoniazide, rifampin and ethambutol (SIRE) was developed and later studies by many workers (Kertcher et al. 1978). An evaluation of this technique was coordinated by the Centres for Diseases Control (CDC) and reported in 1981 (Snider et al 1981). This study was followed by additional refinements by Siddqui (1981). Direct susceptibility testing from AFB smear-positive specimens was also reported in Broman (1982) and was confirmed by other workers (Libonati, 1984, Stager, 1984). Lyophilized primary drugs, streptomycin, isoniazid, rifampin and ethambutol, are available along with a diluting fluid which is used for making a 1:100 diluting of the inoculum for the control.

The BACTEC differentiation test is based on the observations of Eidus, who first reported susceptibility of mycobacteria to p-nitro-a-acetylamino-b-hydroxy-propiophenone (NAP) (Eidus et al 1960, Laszlo and Eidus 1978). Using this principle, Siddiqi Develop a rapid radiometric test to differentiate mycobacteria belonging to then TB complex from MOTT bacilli within 4 to 5 days (Siddiqi 1984 a,b). Laszlo and Siddiqi (1984) reported further evaluation of the
BACTEC NAP test. Other reports have confined the accuracy of the BACTEC NAP test (Gross and Hwakins 1984, Morgan et al. 1985). Development of the NAP test complemented the BACTEC 460 TB Systems, providing rapid isolation, identification and drug susceptibility testing.

The BACTEC NAP test differentiates the TB complex from other mycobacteria. For further speciation, growth on a solid medium is required to carry out biochemical reactions. Two studies were reported indicating that if 12B medium is employed for primary isolation and a subculture is made on a L-J slant at a GI > 100, the growth in this subculture is luxuriant and is achieved approximately at the same time as growth on the L-J slant inoculated from the original specimen (Libonati, 1987). Thus, there will be no loss of time if the BECTEC system is used and a subculture is made on a L-J slant for further identification work. NAP results, however, are obtained much earlier than the niacin test results.

Drug susceptibility testing of *M. tuberculosis* with pyrazinamide (PZA) has always been a difficult task due to the low pH (5.5) at which the PZA susceptibility test was to be performed (Butler and Kilburn 1982). Many isolates of *M. tuberculosis* failed to grow at this pH and thus susceptibility results could not be reported on these cultures. The recent introduction of the BACTEC PZA susceptibility can be tested with reportable, rapid and reliable result (Heifets and Iseman 1985, Salfinger et al. 1989).

The BACTEC 460TB system has been successfully employed for the recovery of *M. avium* complex from AIDS patients (Gill, 1984, Gill et al. 1985; Kiehn and Cammarta 1986). However, the procedure required processing of blood
specimens, which is cumbersome and potentially hazardous. Middlebrook 71113
(BACTEC 13A) medium was developed for isolation of *M. avium* complex from
blood by directly inoculating up to 5 ml of blood into the medium (Siddiqi and
Hwangbo 1986). Field evaluations indicated that this medium isolated
mycobacteria earlier and as efficiently as the concentration method (Strand. 1987;
Witebsky. 1987).

The BACTEC 460TB system offers a simple automated technique with
significant convenience and time saving. It also offers the opportunity to bring
much-needed standardisation into TB bacteriology, allowing laboratory results to
be compared throughout the country. The BECTEC 460TB system offers
techniques and media that are well established through numerous clinical trials and
co-operative studies.