2. Review of literature

2.1 Taxonomy, characteristics and classification of mycobacteria

The genus *Mycobacterium* is the only member of the family Mycobactericeae, which belongs to the order Actinomycetales (Schliesser, 1985). The members of genus *Mycobacterium* share some characteristics, including parts of their antigenic determinants with other members of the order Actinomycetales e.g. *Nocardia, Corynebacterium, Rhodococcus* and *Actinomyces* (Schliesser,1985).

Members of the genus *Mycobacterium* are gram-positive, non-motile, non-endospore-forming, catalase positive, have 0.2-0.6 x 1-10 µm rod shape or occasionally filamentous morphology with the typical “acid-fast” staining characteristic. The optimal growing temperatures vary widely according to different species, from 25°C to over 50°C under aerobic to microaerophilic condition (5-10% CO_2), with generation times from 2 to more than 20 hours. They grow very slowly especially after the first isolation. Most of the members of mycobactericeae are saprophytes and adapt readily to growth conditions on very simple substrates, using ammonia or amino acids as nitrogen source and glycogen as carbon source. Only some of them are facultatively pathogenic (Schliesser, 1985).

The cell wall of mycobacteria contains a high amount of long chain peptidoglycolipid (up to 24% of 60-90 carbon lipids) termed mycolic acid (Wilson and Miles, 1964), to which carbol-fuchsin strongly bind and can not be removed during decolorization with 3% acetic-alcohol solution (Salfinger and Kafader, 1992). Based on their cell wall structure, mycobacteria are very resistible to both chemical and physical influences. In acidic

conditions, for example in 2.5-5% oxalic acid ($C_2H_2O_4$) or 3-6% sulphuric acid (H_2SO_4) at least 30 minutes are needed to inactivate *M.avium*. Mycobacteria can survive in refrigerator condition (4-10° C), in general, more than 6 months without any loss of the ability to multiply. To inactivate mycobacteria by heat at 70 and 65°C for 5 and 15 minutes, respectively, are needed (Schliesser, 1985).

To identify mycobacterial species, Schliesser (1985) proposed 3 groups of criteria to be performed, which are :

1. Optimal temperature for growing and growth rate.
2. Pigment formation, need of oxygen and colony morphology.
3. Biochemical tests and resistance against chemotherapeutic compounds

In general, the following criteria are tested: growth rate at 22°C, 37°C, 42°C and eventually 52°C; photochromogenicity; nitrate reduction; niacine formation; activity of thermoresistent catalase; Tween 80 hydrolysis; tellurite reduction; growth on medium containing 5% NaCl and arylsulphatase activity (Wayne and Kubica, 1986).

Based on etiological data, mycobacteria can be differentiated into four groups. (Schliesser, 1978)

1. *M. tuberculosis* complex, which consists of *M. bovis*, *M. tuberculosis, M.africanum* and *M.microti.* In addition *M.canetti* (Brosch et al., 2002) , *M. pinnipedii* (Counsins et.al., 2003) and *M.caprae* (Aranaz et al., 2003) are now recognized as separated species belonging to this complex.

2. *M. avium-intracellulare* complex , which was first determined as pathogenic in birds.
3. Mycobacteria, which are neither members of *M. tuberculosis* complex nor in MAIC but may be isolated from animals and environmental sources.
4. *Mycobacterium avium* ssp. *paratuberculosis*, which is the causative agent of paratuberculosis or Johne's disease in cattle.

2.2 Occurence and epidemiology

2.2.1 Animal reservoirs

Avian mycobacterioses

Avian mycobacteriosis is a world-wide disease which is caused mainly by the members of *Mycobacterium avium-intracellulare* complex (MAIC) (Thorel et al., 1997), but in Switzerland *M. genavense* is predominant in pet birds (Hoop et al., 1996; Holsboer Buogo et al., 1997). *M. fortuitum, M. tuberculosis, M. bovis* and *M. gordonae* have also been isolated from affected organs of wild and pet birds, although they are less common causative agents (Thoen et al., 1977; Hoop et al., 1996).

Avian mycobacteriosis caused by MAIC has been reported in a variety of poultry species, e.g. pet birds, captive exotic and free-living birds (Thorel et al., 1997; Kauppinen et al., 2001; Tell et al., 2001) and their susceptibilities vary from host species, husbandries and management practices (van der Heyden, 1997; Kauppinen et al., 2001). Most of affected birds are clinically healthy but in some cases poor condition of feathers, signs of anaemia,

cyanotic features of the combs and wattles, distended abdomen, dark greenish-yellow droppings of faeces, palpable granulomatous abdominal mass and liver can be observed. Mycobacterial lesions are usually observed in liver, spleen and intestines (Thorel et al., 1997; Tell, 2001). In advanced cases the infected birds will show pathoanatomic changes of the bones including lytic lesions of diaphyses of long bones, which are detectable by radiography.

The lesions are characterized by greyish-white to yellow granulomas in various sizes from pinpoint to centimetres. The nodules may contain central caseous masses or homogeneous tan or a white center but they are usually not calcified (Bush et al., 1978).

Porcine mycobacterioses

The most frequent causative agents isolated from affected lymph nodes were members of MAIC (Thoen et al., 1981; Dvorska et al., 1999; Fischer, 1999; Komjin et al., 1999).

The MAIC infection in pigs is closely related to contaminated environment or feed, bedding material and soil, but direct contact between pigs seems not to be of major importance as a route of infection (Schliesser, 1985).

Since the eradication programme of *M. bovis* infection in cattle in Germany started in 1950s, the prevalence of *M. bovis* in pigs decreased as well (Schliesser, 1977). On the other hand, the prevalence of MAIC infection has been increasing continuously since 1960s (Kauker and Zettl, 1964; Hellmann, 1966) from then the most important causative agent for

mycobacterial infection in pigs were neither *M. bovis* nor *M. tuberculosis* but the members of MAIC (Schliesser, 1977; Uecker, 1985). Many authors reported about considerable economic losses caused by mycobacteriosis in pigs (Schliesser, 1967; Berthelsen, 1974; Dey and Parham, 1993).

MAIC infection in pig usually causes lymphadenitis with granulomatous lesions in the lymph nodes of head and neck or lymph nodes along the intestines, bronchial lymph nodes may be affected in some cases. Parenchymatous tuberculoid and generalized lesions are rarely found in swine (Thorel et al., 1997; Schliesser, 1985). The prevalence of caseous lesion in swine lymph nodes in Europe varies from country to country. It was reported to be 0.27%, 0.5% and 1.85% in the Czech Republic, the Netherlands and Germany respectively (Fischer et al., 2000a; Komjin et al., 1999; Pavlas, 1998).

Horse mycobacterioses

MAIC infection in horses involves normally the alimentary tract with the clinical signs of weight loss, diarrhoea, anorexia and hypoproteinaemia. MAIC infections may also cause abortion (Cline et al., 1991; Helie and Higgins, 1996), or diffused granulomatous dermatitis (Flores et al., 1991). The guttural pouch infection with chronic granulomatous lymphadenitis of lymph nodes in the cervical region and granulomatous hepatitis has been reported (Sills et al., 1990). MAIC infection in horses may be localized at the skin of head and neck, back regions and also in the ventral part of the body (Flores et al., 1991). A generalized *M. avium*

infection has been reported for the first time in a two-year-old colt in Norway (Gunnes et al., 1995).

Cattle mycobacterioses

As one of the most important zoonotic diseases, bovine tuberculosis caused by *M. bovis* is well-known all over the world. The first clinical symptoms of low fever and coughing with mucous secretion could be detected months or years after infection in most host species. In generalized form various clinical signs may be observed depending on affected organs, for example, weight loss for intestinal tuberculosis or arrhythmia for pericardial tuberculosis. The typical pathoanatomic lesions caused by *M. bovis* are caseous masses with or without calcification in lung, lung lymph nodes and tissues in the thoracic cavity. In generalized cases, lesions in liver, spleen, kidneys, brain, bone marrow and mammary glands could be observed (Schliesser, 1985).

M. avium ssp. *paratuberculosis* is the causative agent of paratuberculosis or Johne's disease in cattle, which is primarily an intestinal infection. Thickening of the intestinal wall with granulomatous enteritis particularly of the ileum and the proximal colon and enlargement of mesenteric lymph nodes can be observed in infected animals with the clinical signs of chronic weight loss and intermittent diarrhoea.

The pathological lesions caused by MAIC infection in cattle, which is indistinquishable from *Mycobacterium bovis*, are mostly confined in lymph nodes along the alimentary tract and respiratory tract (De Lisle et al., 1998).

After the eradication program of bovine tuberculosis, MAIC became of major importance as mycobacteriosis causative agent in cattle. Although MAIC infection does not play a major clinical role in cattle, it is the most common mycobacterial species isolated from bovine granulomatous lymph nodes (Pavlik et al., 2002a). In veterinary aspect, MAIC infection has been considered as one of the main obstacles of eradication programs for bovine tuberculosis all over the world (Schliesser, 1985). Due to close genetic relationship within the taxon *Mycobacterium* the members of this taxon share different antigenic determinants to which animals may have been previously exposed either infected with MAIC or other non-tuberculous mycobacteria (NTM). Therefore extensive cross-reactivity is observed with skin-tuberculin test and other diagnostic methods. False positive results in the indirect anti-IgG ELISA test using whole cell preparations of *M. bovis* as coating antigen, were observed in 39.5% of cattle with NTM infection and in six cattles experimentally infected with MAIC and *M. flavescens* (Auer and Schleehauf, 1988). For the skin-tuberculin test, more than two-thirds of non-specific positive reactions are caused by MAIC (Schliesser, 1985).

Mycobacterioses in non-domestic animals

The members of genus *Mycobacterium* cause infection and disease in a wide range of non-domestic including aquatic animals. Pavlik et al. (2002b) reported cases of *M. bovis* infection in diverse host species both in captured and in free living animals, from red deer (*Cervus elaphus*) to European bison (*Bison bonasus*), from american bison (*Bison bison*) to

reticulated giraffe (*Giraffa camelopardalis reticulata*) and from cassowary (*Casuarius casuarius*) to puma (*Puma concolor*).

MAIC prevalence as a causative agent in non-domestic animals varies from country to country. In Germany, members of MAIC were isolated from more than 10% of non-domestic animals (Moser, unpublished, 2004). Members of MAIC have been isolated from both carvivorous and herbivorous animals such as ferret (*Mustela putorius furo*) (Schultheiss and Dolginow, 1994), european hedgehog (*Erinaceus europaeus*) (Matthews and Mc Diarmid, 1977) and also exotic animals like matschie's tree kangaroo (*Dendrolagus matschiei)* (Montali et al., 1998).

Strains of *M. intracellulare* were isolated from some species of non-human primates with clinical signs of weight loss, anaemia and prostration. Most of them showed macroscopic lesions at lungs- and mesenteric lymph nodes (Fleischman et al., 1982; Goodwin et al., 1988).

2.2.2 The environmental reservoir.

Since the cell wall of mycobacteria contains high amount of lipid, the members of genus *Mycobacterium* are very resistant against chemical disinfectants and dryness. Avian mycobacteria can survive up to nine years in soil, and more than three months in water (Gylstorff, 1987). In mycobacteria-contaminated excretions from lungs or in faeces, mycobacterial cells are viable up to 150 days (Saxer and Vonarburg, 1951).

It is now generally accepted that the contaminated environment plays an important role as reservoir for most NTM, especially for MAIC. Its possibility as the source of infection is widely discussed.

MAIC are recovered in water and soils with low pH, low dissolved oxygen content and high organic burden, MAIC has been isolated not only from fresh water reservoirs but also from salt water sources (Falkinham et al., 1980). Aerosol (Wendt et al., 1980), dust and soil (Kleeberg and Nel, 1973; Portaels et al., 1988), farming environments (Kleeberg and Nel, 1973; Cvetnic et al., 1998; Pavlik et al.,2000) and some species of arthropods and insectivores are also confirmed as MAIC reservoirs (Fischer et al., 2000 b; Fischer et al., 2001; Fischer et al., 2002).

As the most likely source of infection for human beings communal water systems and other water reservoirs have been examined by groups of researchers (du Moulin et al., 1988; von Reyn et al., 1994).

Previous studies in the USA reported the close genetic relationships between MAIC isolates recovered from water and patients with or without HIV infection both in small and large scale studies (Aronson et al., 1999; von Reyn et al., 1994). Two cases of pulmonary MAIC infection in immunocompetent Japanese adults have been reported. Each of the MAIC isolate showed identical IS1245 and IS1311 restriction fragment length polymorphism (RFLP) patterns compared to the isolates recovered from each home bath water system (Watando et al., 2001; Takahara et al., 2002).

Based on conventional and molecular biological methods, 29 strains of MAIC have been isolated from drinking water reservoirs for swine production in the Czech Republic and Slovakia (Pavlik et al., 2000).

The results of many studies suggested that water is a possible source of MAIC infection for humans, although von Reyn et al. reported in 2002 that mycobacterial colonization of home water supplies did not associate with an increased risk of MAIC infection.

2.3 Pathogenesis

Pathogenesis of atypical mycobacterial infection is in general similar to pathogenesis of classical tuberculosis (Schulz, 1987). Although their pathogenicities differ in some respects: *M. avium* is more resistant to effect of the acidic environment in stomach up to pH 2.2 both in exponential and stationary phases (Bodmer et al., 2000) and *M. avium* invades intestinal mucosa primarily by enterocytes (Bermudez et al., 1998) whereas *M. bovis* BCG and *M. avium* ssp. *paratuberculosis* enter intestinal mucosa by using the a specialized cells covering on Peyer's Patches called M cells (Fujimura 1986; Momotani et al., 1988).

Theoretically, mycobacterial infection occurs only when host's immune system is disturbed either locally or generally. Mycobacteriosis starts with the formation of primary granulomas and in advanced cases these granulomas often enlarge until they appear as tumour-like masses called tubercles (Theon and Chiodini, 1993; Ellsworth et al.,1980). It was

found that *Lymphonodus (Ln.) mandibularis* is the most often pig's lymph node affected by *Mycobacterium* followed by both *Lnn. retropharyngei mediales* and *laterales.*

The typical pathoanatomical lesions of lymph nodes in pigs are normally found only along the alimentary tract but generalized lesions may occur when the bacilli travel to other organs via lymph or blood circulation. The acute form of generalization leads rapidly to death. In a chronic form, lesions may become encapsulated and remain in small size without clinical signs with alive microbes (Thoen and Himes, 1986). Necrosis at the centre of granuloma may occur since the circulation system is disturbed by the increasing size of granuloma.

2.4 MAIC infection in humans

Diseases in humans due to MAIC occur primarily in HIV patients with CD4+ cell count below 100 cells/mm^3 of blood (Nightingale et al., 1992; Horsburgh, 1997), other immuno-suppressed individuals and children under 12 years of age. MAIC causes localized lymphadenopathy in the cervical region of children, but cases of otomastoiditis and otitis media have also been reported (Trupiano and Prayson, 2002). In adults with immunodepression, MAIC produces normally three major groups of clinical syndroms: Disseminated disease, pulmonary disease and localized lymphadenitis, mostly in the cervical region (Benson, 1994; Horsburgh, 1997).

Since the 1990s, a dramatic increase of the MAIC infection in humans has been observed. Related to AIDS epidemics, MAIC has been recognized as the most commonly associated systemic infection. Disseminated disease and lymphadenitis have become an

increasingly common clinical problem in many countries (O'Brien et al., 1987; Yates et al.,1993; Pozniak et al., 1996; Tanaka et al., 1997). The incidence of disseminated MAIC infection in HIV patients has been traced in many countries to be 17-50 % (Dawson, 1990; Nassos et al., 1991; Nightingale et al., 1992), but it was rarely found in african patients with AIDS although MAIC have been isolated from water and soils in many countries of the continent. (Morrissey et al., 1992; von Reyn et al, 1993).

MAIC infection in immunocompetent individuals is very rare. In these cases MAIC was mainly recovered in patients with risk factors e.g. smoking, pneumoconiosis, chronic obstructive pulmonary disease and alcohol abuse (Falkinham, 1996).

2.5 Potential zoonotic agent

As a possible source of MAIC infection for humans, pigs have been widely discussed for decades and a number of molecular biological methods have been used for the detection of genetic relationship among isolates.

Komjin et al. reported in 1999 that *M.avium* isolated from pigs and humans share a high degree of IS1245 based polymorphism. Identical band patterns could be obtained in pig and human isolates also by standardized commercial reagent kit for random amplified polymorphic DNA (RAPD) analysis. (Ramasoota et al., 2001)

That means that pig may be an important source of infection for humans or that pigs and humans share common sources of infection.

2.6 Laboratory diagnostic approaches

2.6.1 Microscopic diagnosis

Mycobacterial cells can be detected by light- and fluorescent microscopy. The classical differential stain used to detect mycobacterial cells is the “Ziehl-Neelsen” stain. Due to high content of mycolic acid in the cell wall, the mycobacterial cell shows special characteristics termed “acid fastness“, which means that they retain carbol-fuchsin dye after an 3% acetic-alcohol decolorization step.

The positive result of Ziehl-Neelsen stain shows bright red bacilli in size of 0.2-0.6 x 1.0-10 µm, which should be reported only as “acid-fast bacilli”, since mycobacteria are not the only microorganism which can be stained as acid-fast in a smear. Some species of Nocardia, Corynebacteria, fungi and tissue debris can also be Ziehl-Neelsen positive (Wayne and Kubica, 1986).

Another method for the detection of mycobacterial cells in tissue is the use of a fluorescent microscope to observe specimen stained with fluorochrome e.g. auramine-rhodamine or acridine orange.

Disadvantages of microscopic diagnosis are, that the presence of mycobacteria can be detected only in material, which contains approximately more than 10^4 mycobacterial

cells. And by means of Ziehl-Neelsen stain, viable and dead cells are indistinguishable and do not permit a species identification (Rüsch-Gerdes, 1999).

2.6.2 Isolation of bacteria

Mycobacteria are slowly growing in culture, with a generation time of 2-20 hours compared to 20 minutes for *Escherichia coli*. A decontamination step is necessary to eliminate potentially fast growing contaminating bacteria. Because mycobacteria resist to chemical agents compared to other bacteria, a variety of chemical agents can be used in decontamination step, e.g. 4%NaOH, 20-30% Na_3PO_4, 6% H_2SO_4, 3%HCl. Rüsch-Gerdes proposed in 1997 that N-Acetyl-L-Cystein-NaOH (NALC) is the agent of choice for decontamination. After treatment steps of homogenization decontamination and centrifugation the suspected material is inoculated onto egg containing selective solid media with malachite green as inhibitor for the first isolation.

Mycobacteria can take up to 15 weeks for the first isolation to be recovered on solid media, the isolation of mycobacteria from material using radiometric detection technique is one of the most frequent means of detecting mycobacteria. The principle of this technique is not to detect growth of mycobacteria directly but to detect ^{14}C-marked metabolites. By means of radiometric detection technique, the detection time may be reduced from 3-4 weeks to 7 days. (Hoffner, 1988)

2.6.3 Animal testing

Animal testing for detecting mycobacterial infections normally was used only for *M. bovis* and *M. tuberculosis*, but since decades this technique is no longer be used as routine diagnostic method. It is used only when the suspected specimen contains quite a low amount of mycobacteria and/or mycobacteria can not be isolated from suspected cases.

According to its susceptibility guinea pig is the most suitable animal species for this purpose. Nevertheless, some other rodent species e. g. yellow-brown mice, gold hamster and beige mice are also suitable for the isolation of mycobacteria (Hussel, 1951; Gangadharam et al., 1981). Post mortem examination and also laboratory diagnosis will be performed 6-8 weeks after subcutaneous inoculation.

In the meantime, animal testing is the most realized model for anti-mycobacterial drug testing and immunological study.

2.6.4 Molecular biological identification

In recent years, molecularbiological techniques have been developed and widely used as routine identification procedures because molecular biological techniques provide two advantages when compared with conventional microbiological techniques: a more rapid turn over and an improved specificity of identification. Many of molecular biological methods used based on distinct patterns or sizes of DNA bands, which are obtained by electrophoretic separation of DNA fragments.

Since the basic principle of replication of a piece of DNA using two primers had been described by Kleppe et al. in 1971, the polymerase chain reaction (PCR) has been

developed throughout the years. PCR has provided a highly sensitive and specific diagnostic method by means of amplified nucleic acid detection.

PCR is the recurrent reaction in which the double stranded template DNA is denatured by heating into two single strands. The forward and reverse 20-30 base fragments of DNA complementary to a region of the template, so-called primers, are annealed to the single stranded template by hydrogen bonds. At 72°C , the primer molecules are elongated by the action of a heat-stable polymerase enzyme and produce the complementary copies of templates. After a step of extension all steps will be repeated for 20-40 times, in which the PCR products from earlier cycles act as templates for the following cycles and the heat-stable polymerase enzyme will be reused. By this principle, up to billion copies of DNA can be produced only by 30 cycles.

Sizes of amplified products will be analyzed in horizontal agarose gel electrophoresis. Since DNA has negative charge, they can be moved in agarose gel by being exposed to positive and negative currents and be separated according to their molecular weight. After the step of gel eletrophoresis they will be stained with ethidium bromide and visualized under UV light.

The 16S rDNA sequence which is specific to species level is considered as the molecular gold standard for mycobacterial species identification due to its high degree of conservation. Moreover, the hypervariable region of 16S rDNA serves as target for detection of within-species variation. The method for the identification of genus, species and strains of

mycobacteria at the same time using conserved and variable sequences of the 16S rDNA has been described by Wilton and Cousins in 1992.

The “Multiplex” PCR, which contains more than one primer system, also optimized and described by Wilton and Cousin (1992) for mycobacteria as a rapid, inexpensive and accurate PCR system for the identification of MAIC.

Insertion sequences (IS) are small, genetically compact DNA sequences in bacterial genomes which involve in bacterial translocation and transpose within and between genomes. Their activities can lead to the assembly of gene clusters with special functions. IS have been classified into groups based on their similarity in the primary sequences of their transposases and genetic their organization (Mahillon et al., 1999).

Based on its degree of mobility and high copy of number in MAIC isolated from human and animals, IS1245 has been described as a novel insertion element of MAIC identification (Guerrero et al., 1995).

The study from Bauer and Anderson in 1999 showed that the stability of in vivo IS1245 is high and no change in IS 1245 could be observed after subcultures on solid media for several months but some small changes do seem to occur.

A number of molecular biological techniques has been applied to study the epidemiology of mycobacterial strains. For MAIC pulsed-field gel electrophoresis (PFGE) has been declared as the gold standard for determining genetic relationship among strains (Maslow et al., 1993; Tenover et al., 1995).

PFGE is a technique used to separate fragments of the entire genome by length to generate a distinct pattern of DNA restriction fragment. It operates by the method to extract DNA from agarose embedded bacteria (Schwartz and Cantor, 1984; Hughes et al., 2001). After the steps of detergent-enzyme lysis and digestion the DNA will be separated by alternating eletric fields from a hexagonal array of electrodes to run genomic DNA through agarose gel matrix. Under the alternating electric fields the large DNA molecule will move in first direction for short time and get caught in the gel matrix. When the electric field changes, DNA molecules will move to the other direction. The net movement of DNA which depends on the pulse times of electrodes will be in the forward direction. One of the most widely used enzymes for molecular analysis of MAIC is the enzyme synthesized by Xba I gene from *Xanthomonas badrii, Xba* I, which cleaves at the recognition site of T/CTAGA.

Using this method PFGE allows to separate DNA chains ranging in size from <20 kilobases up to 10 megabases. Compared to PFGE, conventional electrophoresis can only be used for DNA separation in size up to 50 kilobases. The electrophoretic DNA band patterns are visualized by means of ethidium bromide stain and can be analyzed by a variety of computer programmes.

Another powerful molecular biological method for epidemiological studies is the analysis of restriction fragment length polymorphism (RFLP), in which bacterial DNA will be digested by restriction enzymes, which cleave DNA molecules at 4-6 specific base pair recognition sites depending on the particular enzyme used. After the digested DNA will be separated by eletrophoresis, DNA fragments are transferred from agarose gel to special

membrane by blotting (Southern, 1975). The membrane bound DNA fragments can be hybridized and later detected either by enzyme-colorimetric or chemilunescent substrate. Genetic relationship among the samples can be analyzed by a computer program according to the similarity of band patterns.

For MAIC, IS1245 and IS901 are suitable as the target for RFLP (Guerrero et al., 1995; Ritacco et al., 1998; van Soolingen et al., 1998). The most often used restriction enzyme for IS 1245 detection analysis is Pvu II, which is synthesized by gene from *Proteus vulgaris*. Digesting MAIC chromosome by Pvu II restriction endonuclease yields the restriction fragmensts in size from 0.5-20 kb by recognizing and cleaving the recognition sequence CAG/CTG.

Some other technique, for example high-performance liquid chromatography (HPLC) has been described as a fast and efficient method by analyzing mycobacerial mycolic acid patterns (Butler et al., 1992). However, the HPLC equipment is costly and needs substantial numbers of samples to maintain the system.