

DETERMINATION OF ANTIBIOTIC PATTERN OF MYCOBACTERIUM SPECIES FROM AQUATIC SOURCES AND ITS INSILICO COMPARISON WITH CLINICAL SAMPLES

Submitted in partial fulfillment of the requirements for the award of Master
of Technology degree in biotechnology

by

HARITHA.S (41810005)



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119

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This is to certify that this Project Report is the bonafide work of **HARITHA.S** (41810005) who carried out the project entitled "**DETERMINATION OF ANTIBIOTIC PATTERN OF MYCOBACTERIUM SPECIES FROM AQUATIC SOURCES AND ITS INSILICO COMPARISON WITH CLINICAL SAMPLES**" under our supervision on May 2023

Internal Guide

Dr. M. BAVANILATHA

External Guide

Dr. KRUPAKAR PARTHASARATHY

Head of the Department

DR. V. RAMESH KUMAR

Submitted for Viva voce Examination held on 05/05/2023

Internal Examiner

External Examiner

DECLARATION

I HARITHA.S (41810005) hereby declare that the Project Report entitled **“DETERMINATION OF ANTIBIOTIC PATTERN OF MYCOBACTERIUM SPECIES FROM AQUATIC SOURCES AND ITS INSILICO COMPARISON WITH CLINICAL SAMPLES”** done by me under the guidance of **Dr. M. Bavanilatha (Internal)** and **Dr. Krupakar Parthasarathy (External)** in Centre for Drug Discovery and Development at Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Master of Technology degree in Biotechnology.

DATE: 5/5/2023

PLACE: Chennai


SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of SATHYABAMA for their kind encouragement in doing this review and for completing it successfully. I am grateful to them.

I convey my thanks to Dr. V. Ramesh Kumar, School of Bio and Chemical and Head of the Department, Dept. of Biotechnology for providing me with necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide Dr. M. Bavanilatha and Dr. Krupakar Parthasarathy for their valuable guidance, suggestions and constant encouragement that paved way for the successful completion of my project.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology and Centre for Drug Discovery and Development who were helpful in many ways in the successful completion of the project.

ABSTRACT

Mycobacterium is a unique genus in the family Mycobacteriaceae and a saprophytic microbe that belongs to the order Actinomycetales. Since these bacteria have a 20-hour growth cycle, isolation and identification could take up to 6 weeks (although a few species may grow in only 5–7 days). Mycobacteria are categorized into three classes based on their clinical importance. Class I Mycobacteria are virulent forms that infect humans and animals and cause disease. Class II and II are other Tuberculosis causing bacteria and atypical mycobacteria. The natural habitat of these secondary disease-causing mycobacteria is the aquatic environment. The present study is to evaluate the antibiotic activity against Mycobacterium from aquatic sources. Water and sand are the selected samples used for isolation. Gram stain and zeihl neelsen stain are the stainings used to detect the mycobacterium initially. Specific mycobacterial species are determined through biochemical testing. Extracted DNA is sequenced to identify the mycobacterium at the species level. The result of this study is to test the drug sensitivity and determine whether a similar medicine will be used to treat clinical isolates of mycobacterium. The future work of this study is to evaluate the protein stability of selected species and also, examine the level of mutation in their DNA by in-silico analysis. This will be beneficial for further therapeutic development.

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SYMBOLS AND ABBREVIATION

MB 7H11 Agar	-	Middlebrook 7H 11 Agar
MB 7H9 broth	-	Middlebrook 7H9 broth
ADC	-	Albumin bovine serum, Dextrose, and catalase
Mcg	-	Microgram
Cm	-	Centimeter
µl	-	Microlitre
nm	-	Nanometer
ml	-	Milli liter
g	-	Gram
L	-	Litre

Chapter 1

INTRODUCTION

Mycobacteria are classified as typical *Mycobacterium* consisting of *M. tuberculosis* complex, which is nearly absent from the environment, Nontuberculous *Mycobacterium* (NTM), and saprophytic species (Schulze-Röbbecke & Buchholtz, 1992). *Mycobacterium* of this saprophytic category is extensively spread and present in dust, water, and soil. However, they rarely harm people.

Mycobacterium is a genus and exists in the *Mycobacteriaceae* family. It is recognized to afflict humans with disease (Puttinaowarat et al., 2000). *Mycobacteria* occur in systems that provide water (Schulze-Röbbecke & Buchholtz, 1992). The waxy cell membrane is the most well-known virulence element of the *mycobacterium* genus, which provides resistance to drugs and host immune systems and helps in surviving in water (Honda et al., 2015).

Atypical mycobacteria are acid-fast bacilli rarely detected in humans associated with a lung infection, a respiratory disease that resembles *M. tuberculosis*. It is classified by their colonial morphology and studies conducted in patients affected with Nontuberculous mycobacteria (Timpe & Runyon, 1954; Crow et al., 1957). The development and spread of the disease are probably aided by NTM-specific virulence determinants. The apolar lipids, glycolipids, phospholipids, and amphipathic lipids are the four primary lipid classes that are present in the NTM envelope. NTM-derived lipids alter the host immunological responses, which helps pathogenesis (Honda et al., 2015). NTM can synthesize glycopeptidolipids (GPL) which is crucial for biofilm development and sliding character (Enomoto et al., 1998; Freeman et al., 2006). Results found that 1.78 cases were affected by NTM in the United States (Schulze-Röbbecke & Buchholtz, 1992).

NTM are opportunistic mycobacteria that can infect humans, animals, and fish with the disease. They are saprophytes in their natural habitat (Falkinham, 2002). Despite the identification of over 160 different NTM species, only a few species are responsible for the majority of infections and it is found in soil as well as water sources (Edirisinghe et al., 2014). In an oligotrophic water environment, *M. xenopi*, *M. marinum*, *M. scrofulaceum*, *M. chelonae*, *M. kansasii*, *M. phlei*, *M. intracellulare*, *M. fortuitum*, and

M. avium complex were isolated to test their heat sensitivity (Schulze-Röbbecke & Buchholtz, 1992). Water samples obtained for testing revealed the presence of the species *M. marinum*, *M. phlei*, *M. fortuitum complex*, *M. gordonae*, and *M. scrofulaceum* (Edirisinghe et al., 2014). *M. xenopi* and *M. kansasii* species were determined in the samples of drinking water (Falkinham, 2002). Water is disinfected with chlorine to prevent mycobacterial colonization, although some mycobacteria are resistant to chlorine, making this method ineffective (Schulze-Röbbecke & Buchholtz, 1992). By using reverse cross blot hybridization polymerase chain reaction, *M. marinum* and *M. fortuitum complex* were found in the water as well as fish samples acquired from Thailand fish farms (Puttinaowarat et al., 2000).

Rather than spreading from a human or an animal, organisms that trigger human disease impact them after exposure to the environment (Edirisinghe et al., 2014). Travelers are mainly suffering from skin disorders like Skin and Soft tissue Infections caused by bacterial and other mycobacterial species (Diaz, 2014). Workers in fish farms were observed to have cutaneous lesions and nodules due to aquatic mycobacteria's existence (Puttinaowarat et al., 2000). Various sources of mycobacterial transmission from aquatic systems to humans are demonstrated in fig 1.

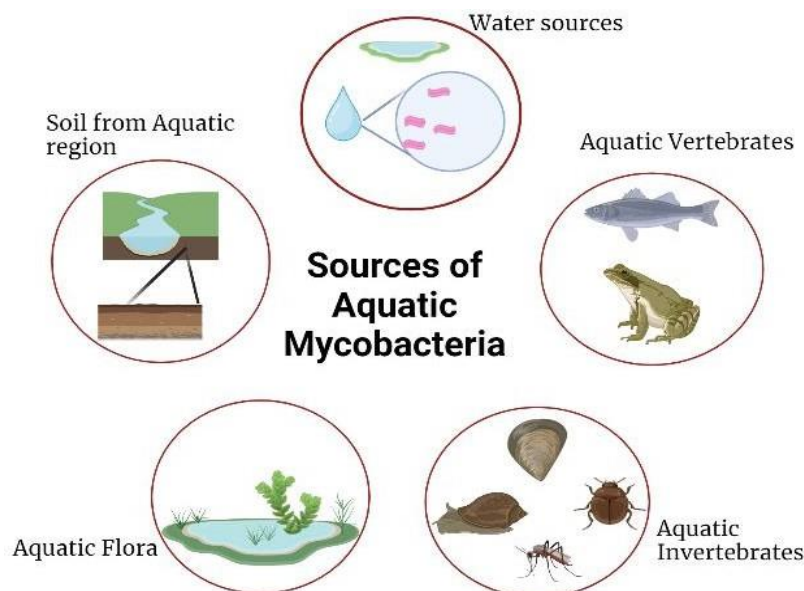


Figure 1.1: Various Sources of Transmission of Aquatic Mycobacteria to Humans

Aquatic mycobacteria thrive in freshwater and marine habitats with temperatures between 30°C and 33°C (Diaz, 2014). The cause of fish Mycobacteriosis is aquatic mycobacteria. It also functions as a human pathogen and is not just pathogenic to fish (Puttinaowarat et al., 2000). A case study from 2012 highlighted that certain pathogens may produce comparable infections. Therefore, to determine the mycobacterial species, tests such as the QuantiFERON-TB Gold test, tuberculin skin test, acid-fast staining test, and PCR detection are used (Diaz, 2014). This article highlighted the necessary antibiotic regimen for treatment in order to prevent the propagation of mycobacterial infections in the following.

Chapter 2

REVIEW OF LITERATURE

2.1 Mycobacterial Species

2.1.1 *Mycobacterium marinum*:

Initially, *Mycobacterium marinum* was named in 1926 after the disease was identified in saltwater fish. It was again identified by Baker and Hagan in 1942, they named it *Mycobacterium platypoecilus* caused tuberculosis in Mexican platyfish. In 1949, Besse discovered species named *Mycobacterium anabantid*, and *Mycobacterium balnei* was named by Nordan and Linell in 1951. Later all these strains were considered as same species due to their morphology, and pathogenic data and named *Mycobacterium marinum* (Wolinsky, 1979; Gauthier & Rhodes, 2009; LeBlanc et al., 2012). The causative organism was identified after several people had experienced tuberculoid infection and granulomatous skin lesions after using the public swimming pool in Sweden (1939) and the United States (1951). Due to its relationship to home aquaria and associated activities, the disease created by *M. marinum* which was previously known as swimming pool granuloma is now known as fish handler's disease and fish tank granuloma (Aubry et al., 2017).

16s rRNA sequence of *M. marinum* shows that the species lies in the genus which is closely similar to the branch that consists of *Mycobacterium tuberculosis*. Infection with *M. marinum* usually appears in the hand and even rarely occurs in the liver, eyes, and lungs. Deeper infection including arthritis, osteomyelitis, and tenosynovitis is specifically connected with water-related activities (Wayne & Sramek, 1992). *M. marinum* causes red spots in the skin at the inoculation point. After some days, it will develop single or multiple nodules at the inoculation point and become a severe ulcerate infection (Diaz, 2014). A study reported that this strain was nonresistant to kanamycin, pyrazinamide, ethionamide, and viomycin, but resistant to isoniazid and streptomycin (Wolinsky, 1979). Combined forms of antibiotics such as rifampin and ethambutol or clarithromycin and ethambutol are recommended for people infected with *M. marinum*, even chemotherapy, and surgery are required to treat the infections. Even though individuals have positive for HIV and AIDS infection, superficial lesions

can be treated with insufficient evidence. An increase in infection develops awareness and improves frequent diagnosis as well (Diaz, 2014; Wayne & Sramek, 1992).

2.1.2 *Mycobacterium ulcerans*:

M. ulcerans, a newly evolved strain derived from *M. marinum*, is the agent responsible for causing Buruli ulcers. It was discovered in 1948 and named *Mycobacterium ulcerans* after the characterization of the disease. It is a slow grower and has a suitable growth temperature of 33°C. This species is identified in salivary glands and tissues of aquatic insects (Naucoridae, Belostomatidae, Hydrophilidae, Corixidae, Ranatridae, Nepidae, and Hemiptera) and animals (Planorbidae, Mollusks, and *Hemichromis bimaculatus*). Also, the DNA of *M. ulcerans* was detected in the stem and leaves of aquatic flora (*Vicia torta*, *Vicia nana*, *Crinum calamistratum*, and *Eriocephalus africanus*) (Zingue et al., 2018).

This organism contains pMUM virulence plasmid which carries MLS A1, MLS A2, and MLS B genes responsible for producing polyketide synthase. This enzyme synthesizes a lipid toxin called mycolactone described as a heat-labile substance that gives a cytopathic effect on cells in tissue culture and causes severe ulceration on subcutaneous tissue when inoculated in the culture into the skin (Wolinsky, 1979; Tan et al., 2020).

M. ulcerans cannot penetrate into a human through intact skin from an external source. Thus, the transmission of bacteria to humans through skin contamination with insect bites or direct contact with infection (Zingue et al., 2018). Exposure to mosquitoes raises a person's risk of developing a Buruli ulcer (Stinear & Johnson, 2008). There are several other names for the Buruli ulcer including Bairnsdale, Daintree, and Mossman (Johnson et al., 2007). The three clinical stages of Buruli ulcer in humans: are pre-ulcerative lesions, ulcerative lesions, and scars determined based on usual appearance (Zingue et al., 2018). An effective preventive measure is to avoid skin contamination by introducing clothing in endemic areas. Buruli ulcer was detected in 33 countries including Africa, South America, and Asia. The prevalence of the disease increased mostly in Africa between 1970 to 2010. However, rapid measurements including laboratory confirmations with Ziehl-Neelsen staining, molecular detection method, histopathological analysis, MALDI-TOF-MS, and Fluorescent thin layer chromatography technique are recommended to decrease the prevalence. A

combination of oral clarithromycin with rifampin is used for treatment. Early detection of Buruli ulcers in patients can be cured by an antibiotic or skin graft method (Zingue et al., 2018).

2.1.3 *Mycobacterium fortuitum*:

Mycobacterium fortuitum is a nonchromogenic and rapidly growing mycobacterium identified from soil and water. It commonly affects immunosuppressed patients and causes pulmonary infection, soft tissue, and bone infection (Okamori et al., 2018). The presence of *M. fortuitum* strains in the water may have caused nosocomial infections (Burns et al., 1991). Species classification is important for epidemiologic studies. The *M. fortuitum* complex consists of two separate species such as *M. fortuitum* and *M. chelonae*. *M. fortuitum* was isolated by positive iron uptake and nitrate reduction biochemical tests. A report described that *M. fortuitum* species are further classified into subspecies are *bio-variant fortuitum* and *biovar peregrinum* (Lévy-Frébault et al., 1983; Silcox et al., 1981). This strain is effectively resistant to many Anti tuberculous and antimicrobial drugs (Okamori et al., 2018).

2.1.4 *Mycobacterium chelonae*:

A rapidly proliferating NTM known as *Mycobacterium chelonae* is categorized as Runyon group IV. It requires less than 7 days for visible growth in the diluted inoculum (Wayne & Sramek, 1992). *M. chelonae* is presented widely in water, dust particles, soil, and aquatic animals (Preda et al., 2009).

In 1903, Freidmann isolated *M. chelonae* from a turtle and named it turtle tubercle bacillus (Akram et al., 2017). *M. chelonae* is further subdivided into *M. chelonei subsp. abscessus* and *M. chelonei subsp. chelonei* (Lévy-Frébault et al., 1983). Nonchromogenic *M. abscessus* and *M. chelonae* were observed as the same species, later it was confirmed that *M. chelonae* was differentiated from *M. abscessus* due to their nonidentical gene sequence and hsp65 (Akram et al., 2017). Normally, cutaneous infection in humans is uncommon. Organisms frequently cause dermal lesions when associated with skin wounds and acupuncture or tattooing. Lesions are characterized as red violaceous nodules. Rarely associated with Clinical illness including osteomyelitis, ocular disease, and pulmonary infection (Preda et al., 2009). *M. chelonae* causes disseminated infection in patients suffering from cancer, chronic kidney disease, and immunocompromised diseases (Akram et al., 2017).

Infection with *M. chelonae* is more difficult to treat because it is strictly resistant to cefoxitin, rifampicin, imipenem, amikacin, and minocycline. Moderate susceptibility with clarithromycin, tobramycin, azithromycin, and moxifloxacin. A previous case study reported that an old man was diagnosed with *M. chelonae* after he had several cutaneous lesions and he was recommended to take clarithromycin for symptom reduction (McCallum & Johnson, 2016).

This species is mostly resistant to all antimicrobial agents therefore dual therapy is recommended for treatment. It often takes several months to recover. In an early study, clinicians used line probe assay, High-Performance Liquid Chromatography, and agar disk diffusion technique for identifying the rapidly growing *M. chelonae* from the specimen. The dermatologist has suggested clarithromycin and moxifloxacin for *M. chelonae* infection (Preda et al., 2009).

2.1.5 *Mycobacterium pseudoshottsii* and *Mycobacterium shottsii*:

M. shottsii was not detected in any samples collected from the Chesapeake Bay and Rappahannock rivers (Gauthier et al., 2010). Then it was observed in the splenic tissue of striped bass (Gupta et al., 2013). whereas *M. pseudoshottsii* was initially discovered in the granulomatous lesions of striped bass and white perch. Additionally, it was identified in DNA extracts from water, sediments, and tissues of striped bass target species in both rivers by real-time PCR (Mugetti et al., 2020).

Due to the similarities in their gene sequences, Slow-growing *M. shottsii* and *M. pseudoshottsii* are strongly correlated and they may be distinguished when they produce pigments (Gauthier et al., 2010). These *Mycobacterium* species have been demonstrated to be quite similar to *M. ulcerans* and *M. marinum* by 16srRNA sequencing (Rhodes et al., 2003). *M. pseudoshottsii* has an insertion sequence and the potential to produce the mycolactone toxin that *M. ulcerans* does. *M. shottsii* was not demonstrated that produced any toxin (Gauthier et al., 2010).

2.1.6 *Mycobacterium avium* complex:

The most significant ailment affecting domestic birds is avian tuberculosis. The cause of avian TB may be attributed to a number of mycobacterial species. *Mycobacterium avium* is mostly responsible for the sickness. Avian tuberculosis can also be brought on by certain species including *M. tuberculosis*, *M. scrofulaceum*, *M. intracellulare*, *M. bovis*, and *M. fortuitum* but these cases are extremely uncommon. *M. avium* likely

infects every species of bird and produces avian tuberculosis. Disease susceptibility varies between species (Dhama et al., 2011).

In addition to immunocompromised people, *Mycobacterium avium complex* consists of *M. intracellulare*, and *M. avium subsp. hominissuis*, *M. avium subsp. silvaticum*, *M. avium subsp. avium* and *M. avium subsp. paratuberculosis* can affect various species of animals including cattle, goats, dogs, horses, cats, and other tropical species (Stear, 2005).

Western Germany has researched over 100 human cases of the disease brought on by *M. avium complex* species between the years 1955 and 1975 (Meissner & Anz, 1977). The agglutination test is among the most popular techniques for identifying and categorizing mycobacteria (Schaefer, 1965). The initial studies were conducted in 1926 to evaluate the serologic characteristics of the *Mycobacterium avian complex* using complement fixation and also agglutination. Researchers in that study were able to determine the various avian serotypes in lepromatous lesions (Furth, 1926). The *M. avium complex* is fundamentally separated into two groups namely serotypes 1, 2 as well as 3 constituting the actual *M. avium* organisms whereas the rest 18 serotypes are regarded as indicative of *M. intracellulare* (Schaefer, 1965). Based on the level of disease pathogenesis, 21 serotypes are subdivided into three different groups that are described simply as the *avian* group, intermediate group, and *intracellular* group (Anz et al., 1970).

M. avium strains found from water samples taken in a shower matched the DNA of *M. avium* specimens from patients with pulmonary illness (Falkinham III et al., 2008). Humans who are exposed to and come into touch with natural water sources like lakes, rivers, and water distribution including swimming pools, become infected with *M. avium* (Ichiyama et al., 1988; Lumb et al., 2004). Four more subspecies of *M. avium* have been identified: *M. avium subsp. hominissuis*, *M. avium subsp. paratuberculosis*, *M. avium subsp. silvaticum*, and *M. avium subsp. avium* (S.-Y. Kim et al., 2017). *Mycobacterium avium subsp. hominissuis* (MAH) primarily infects humans and pigs. It is a common environmental bacterium present in water, dust, straw, and soil (Scherrer et al., 2018). It is responsible for the majority of human cases of *M. avium* disease (S.-Y. Kim et al., 2017). Lymphadenitis in children's head and neck regions, which requires

surgical intervention for treatment, is another typical symptom of *Mycobacterium avium* infection (Agdestein et al., 2014; Bruijnesteijn van Coppenraet et al., 2008).

As mycobacteria may persist in the atmosphere over many months, afflicted birds, polluted water, and contaminated soil are important sources of disease transmission. Areas with dense populations, poor hygienic environments, and unsanitary circumstances are associated with higher disease prevalence. It is highly advantageous for tuberculosis to spread when breeders are kept for a number of years and birds are allowed to fly freely. *M. avium* is known to cause disseminated infection, respiratory illness, and lymphadenitis in people. These diseases are progressive and resistant to antibiotic therapy. Therefore, proper care should be used when handling diseased birds on farms and live *M. avium* cultures in the research laboratory (Dhama et al., 2011).

2.1.7 *Mycobacterium gordonae*:

This mycobacterium is identified as an infectious frog pathogen after causing subcutaneous nodules in a colony of African clawed frogs (Sanchez-Morgado et al., 2009). Amphibian Mycobacteriosis affects the integument as well as the spleen, liver, skin, respiratory and digestive tract that manifests as many grey lumps of different sizes in each of these organs (Wright & Whitaker, 2000).

The infrequent human pathogen *M. gordonae* has been linked to dermatological complications and granulomatous infections, particularly in immunocompromised individuals (McIntyre et al., 1987; Bernard et al., 1992). *M. gordonae* species were one of the numerous mycobacteria which produced the instances (3%) of non-Tuberculous infections among AIDS patients that were not caused by *Mycobacterium avium* complex (Lessnau et al., 1993). Bone marrow and sputum samples from AIDS patients were used to isolate the *M. gordonae* strain (Bagarazzi et al., 1996). occurrence of a woman with HIV who was receiving ethambutol-based antimycobacterial therapy when she contracted a cutaneous illness caused by *M. gordonae*. A definitive diagnosis was suggested based on colony isolation and DNA probe typing that showed the existence of the *M. gordonae* strain. The mycobacterial sensitivity pattern indicated rifampin, isoniazid, and streptomycin resistance (Rusconi et al., 1997).

M. gordonae can be effectively established to induce a number of clinical disorders including tenosynovitis, osteomyelitis, pneumonitis, and other soft tissue infections,

even if its isolation in clinical samples does not necessarily suggest disease (Kurnik et al., 1983). Clarithromycin, fluoroquinolones, rifabutin, ethambutol, and cycloserine have been consistently shown to be effective in the laboratory towards *M. gordonae* species (Brown et al., 1992). Almost half of the examined isolates were resistant to kanamycin, streptomycin, rifampin, clofazimine, and ethionamide, however, a small percentage of isolates were responsive to pyrazinamide and isoniazid (Weinberger et al., 1992).

2.1.8 *Mycobacterium kansasii*:

NTM transmission from person to person is relatively uncommon, but there is a possibility of the transfer of some NTM species, including *Mycobacterium kansasii* (Monde et al., 2018). A case study was not able to demonstrate a shared household exposure since the *M. kansasii* strains collected from the two individuals proved genetically similar (Ricketts et al., 2014).

M. kansasii is most frequently seen in the water and persists for a number of months, While it is soon destroyed in soil (Joynson, 1979). Water tanks and piping systems are prominent with mycobacteria. *M. kansasii* is just similar to other nonmotile mycobacteria and has the ability to build up in a biofilm that forms at the solid-liquid contact inside pipes. 90% of a suspension's viability can be lost in six minutes at 55°C temperature (Dailloux et al., 1999).

M. kansasii is one of the final species to separate from a lineage before the *M. tuberculosis* complex appeared and is able to cause an intense and devastating lung disease that resembles tuberculosis (Luo et al., 2021).

Clinical strains and water isolates are typically genetically dissimilar. According to molecular typing, *M. kansasii* contains a minimum of six different subtypes each of which has a different proliferation and clinical importance (Zhang et al., 2004). *M. kansasii* complex comprises *Mycobacterium kansasii*, *Mycobacterium persicum*, *Mycobacterium pseudokansasii*, *Mycobacterium ostraviense*, *Mycobacterium innocens*, *Mycobacterium attenuatum*, and *Mycobacterium gastri* (Jagielski et al., 2020). Subspecies should be more correctly referred to as species with close affinities, this was suggested on the basis of genome-wide average nucleotide identity (Tagini et al., 2019).

2.1.9 *Mycobacterium phlei*:

Mycobacterium phlei, which is typically thought of as a non-pathogenic organism, has been linked in one case to chronic ambulatory peritoneal Dialysis–related peritonitis. A patient who frequently experienced episodes of peritonitis with a culture that was negative had this microorganism recovered from their peritoneal fluid. The Tenckhoff catheter was removed, hemodialysis was started and cefoxitin, doxycycline, and amikacin were used as antimycobacterial medications as all part of the treatment plan, which was premised on prior observation with fast-growing another atypical mycobacterium (Paul & Devarajan, 1998). A 7-year-old child had knee effusion, conjunctivitis, and urethritis as signs of Reiter's condition. The *M. phlei* was found during knee aspiration. A nine-month prescription of rifampin, streptomycin, and isoniazid helped him get rid of his problems (Aguilar et al., 1989).

M. phlei is a member of the saprophytic species and falls under the category of rapidly developing species. A healthy guy without a history of trauma or vaccination was infected with *M. phlei* in the posterior tibial tendon and flexor digitorum longus tendon. A thorough histologic investigation, anaerobic, fungal, aerobic, and acid-fast bacteria cultures, along with gadolinium-enhanced Magnetic resonance imaging were used to diagnose this infection. It was effectively cured with vigorous surgical treatment combined with a two-month regimen of clarithromycin and ciprofloxacin (Spiegl & Feiner, 1994).

2.1.10 *Mycobacterium scrofulaceum*:

There are few documented instances of this pathogen causing lung diseases or any extranodal involvement (Gracey & Byrd, 1970). However, Human illnesses that are certainly brought on by this mycobacterium are hardly reported other than cervical lymphadenitis in children (Lincoln & Gilbert, 1972; Korsak, 1975).

There have only been 11 recorded cases of widespread *M. scrofulaceum* infection to date, and the majority of certain cases have often been linked to AIDS and other related immunodeficiency diseases.

An HIV-positive patient has been documented to have an instance of widespread *M. scrofulaceum* contamination which manifested as persistent nodular and severe

ulcerative cutaneous infections with possible cavitary pulmonary disease. The combination of ethambutol and rifampin as well as clofazimine, clarithromycin, and ethambutol were all found to be effective against this isolate. All other medications that were examined had no effect on them (Sanders et al., 1995).

The case described in the paper involved an initially healthy person who developed an infection with *M. scrofulaceum* and displayed granulomas in the liver, miliary nodules in the lung, bone inflammation, mediastinal lymphadenitis, skin abscess, and renal disease. A number of abscesses, sputum, and urine samples were used to isolate *M. scrofulaceum*. Ethambutol, ofloxacin, rifampin, and isoniazid were used in conjunction to treat the patient effectively (Hsueh et al., 1996).

Due to the disease's prevalence and the aggressiveness of underlying conditions, the conventional clinical course for *M. scrofulaceum* disseminated infection is debatable and it is challenging to assess the treatment's effectiveness (Wolinsky, 1992; Woods & Washington, 1987).

2.1.11 *Mycobacterium xenopi*:

An organism discovered in skin lesions of *Xenopus laevis* called *Mycobacterium xenopi* was initially identified in 1959 (Schwabacher, 1959). *M. xenopi* was primarily identified under a Runyon group III nonchromogen and has a number of biochemical characteristics with other members in that group, most notably the *M. avium-intracellulare complex* (Engbaek et al., 1967).

M. xenopi grows slowly at 37°C and best around 42°C since it is thermophilic. Before mycobacterial colonies were visible, cultures might have to be incubated for 12 weeks at 37°C. If acid-fast bacilli stain is positive but there is not any proliferation after incubation at 37°C for 8 weeks, *M. xenopi* must be considered (Köhler, 1975). The therapeutic efficacy of *M. xenopi*'s isolation in the lab is not widely known among physicians. Although *M. xenopi* was found in a variety of animal species, its native environment has yet to be identified (Damsker et al., 1982).

Even though *M. xenopi* disease can affect normal individuals, the person diagnosed with *M. xenopi* lung infection is a man who has a history of pulmonary infection. The infection typically progresses slowly and chronically. Even as nodular lesions that looked like tumors were also found, cavitary illness affected the majority of the individuals. Bone, epididymis, lymph nodes, and a sinus tract have all been

documented as sites of extrapulmonary infection (Marks et al., 1975; Grange et al., 1982). Human lung disease instances have been reported sporadically, and water systems in hospitals being colonized have been linked to nosocomial infections (Dornetzhuber et al., 1982; Gross et al., 1976).

Sensitivity analyses in vitro suggest that drug combinations such as streptomycin, ethambutol, rifampin, or ethionamide might be appropriate. Although erythromycin is potent in vitro, the effectiveness of erythromycin in mycobacterial infections is unknown. To control the disease condition and minimize the recurrence, medication should probably be sustained for 18-24 months (Simor et al., 1984).

Chapter 3

AIM AND OBJECTIVES

AIM

To Isolate and Characterize the Mycobacterium from aquatic source and identify its antibiotic pattern

OBJECTIVES

- To isolate the mycobacterial culture from the sample based on their growth and pigment.
- Confirm the mycobacterium with a motility test, Acid-fast stain, and other biochemical assays.
- To analyze the mycobacterium using extracted DNA at the species level by sequencing.
- To perform the drug sensitivity test for the detection of an antibiotic pattern of mycobacterium
- To evaluate the protein stability of isolated culture with clinical samples by In-silico analysis for a comparative study.

Chapter 4

MATERIALS AND METHODS

4.1 Sample collection

Sand and water samples were collected from the seashore of Kovalam, kaasimedu, and Goa. Each sample was collected in a 50ml sterile centrifuge tube and stored at 4°C temperature for further processing.

4.2 Isolation of Mycobacterium

4.2.1 Sample preparation

Two grams of sand samples were suspended in 10 ml of distilled water. The solutions were shaken for 10mins and allowed to settle down. Supernatants were collected from each sand sample and used for inoculation. Water samples were shaken for 2mins before being inoculated onto a plate.

4.2.2 Plating

Mycobacterial colonies were cultivated by the spread plate method. 100µl of both water and sand samples were added individually onto a Petri plate containing 100 ml of Middlebrook 7H11 agar with 0.1mg malachite green and 10 ml of ADC supplement added. Samples were distributed throughout the Petri plate using L-rod and covered the plate with parafilm and kept for incubation.

Inoculated sample plates were incubated at 30°C and 37°C. Rapid-growing organisms take less than 7 days and slow-growing organisms consume more than 7 days for growth. Colony growth can be calculated by daily surveillance.

4.2.3 Subculturing

The cultivated colonies were subcultured onto fresh Petri plates with MB 7H11 agar by quadrant streaking method in order to achieve pure culture and individual colonies were isolated from the plate. Also, each individual colony were inoculated in MB 7H9 broth and cultured for further examination.

4.3 Characterization and Identification of Mycobacterium

4.3.1 Colony Differentiation

Generally, Mycobacterium can be classified by their growth and pigment. Organisms that grow within seven days are referred to as Rapid growers whereas organisms that take more than a week to grow are called Slow growers. The growth of each colony was individually measured via regular observation. Also, mycobacterium produces pigment according to the laboratory environment. Photochromogens are organisms that exhibit color after exposure to a light source. The colonies that produce color while growing in the dark and after light source are called Scotochromogens. However, Nonchromogen organisms usually produce non-pigmented colonies in both dark as well as light environments. In this work, colonies with distinct phenotypic characteristics were noted, and determine the color that colonies produce during their growth for Organism identification.

4.3.2 Staining

4.3.2.1 Gram Staining

Gram staining is the most significant and fundamental technique to identify the kind of bacteria whether it is gram-positive or gram-negative based on the permeability and thickness of their cell walls. The loss of the crystal violet stain from thin-walled gram-negative cells makes them appear red as a result of the counterstain. Gram-positive cells have strong walls and low levels of lipids, which gives them their blue appearance. It is quite hard to stain Mycobacterium using the gram-staining method because it has a complicated cell wall structure, which is made up of the phosphatidyl-myoinositol-based lipo glycans and mycolyl-arabinogalactan-peptidoglycan complex. Mycobacterium is typically regarded as gram-positive bacteria.

4.3.2.2 Acid-fast Staining

Mycobacteria can be recognized by Acid-fast stain due to their unique acid-fast cell wall, which is different from that of other bacteria. The isolated colonies were transferred from Middlebrook 7H11 agar into 7H9 broth and incubated for the staining procedure. A sterile disposable loop was used to spread the colony onto a glass slide, and the slides were left to air dry for five minutes. After the culture had been air-dried, it was fixed with heat. Ziehl Neelsen stain procedure was carried out using 1% carbol

fuchsin, 20% sulfuric acid, and 0.1% methylene blue. Under a microscope, pink to red color represents acid-fast organisms, while blue color denotes non-acid-fast bacteria.

4.3.3 Motility Test

This test can be done in two ways such as the microscopic method and the Biochemical method. The hanging drop method is a common microscopic method used in this study for checking the motility of bacteria and this technique can be performed using fresh culture broth. The culture was inoculated into MB 7H9 broth solutions and incubated for 24hrs. Middlebrook 7H9 broth was prepared by adding 10ml of ADC supplement in 90ml of diluted MB 7H9 broth. The culture will be observed under the microscope for bacterial motility.

4.3.4 Biochemical Assays

Biochemical tests were performed to differentiate and identify the organisms. Both positive and negative results are important and noted for classification. Tween 80 hydrolysis and urease tests were carried out in this study. The culture was placed into tween 80 substrate from a solid MB 7H11 agar plate. Tween 80 substrate was prepared in a test tube by adding 100 μ l of tween 80 solutions in 2ml of sterile distilled water and kept in a boiling water bath at 50°C until it dissolved. For the urease test, 0.4 g agar solution was sterilized by autoclave at 121°C and ingredients (peptone, dextrose, sodium chloride, potassium phosphate monobasic, urea, and phenol red) were measured and filter sterilized by 0.45 μ m. These ingredients were added into cooled agar solutions and poured 5ml into each test tube as a slant method. Measure the color change of culture after incubation.

4.4 Genomic studies

4.4.1 Isolation of DNA

4.4.1.1 Sample Preparation

MB 7H9 broth was freshly prepared for 50ml for sample inoculation. Colonies were transferred to each Sterile test tube containing 5ml of broth. The culture tube was mixed via a vortex for 2mins and the solutions were transferred into a microcentrifuge tube.

4.4.1.2 DNA Extraction

The thermolysis method was used for extracting the DNA from mycobacterium culture. A Microcentrifuge tube that had culture was placed in a 100°C water bath to break the cell membrane for 3-4mins. The suspensions were centrifuged at 4°C for 10mins at 8000rpm. Supernatants were collected in a sterile tube and analyzed the DNA concentration using UV spectrometry.

4.4.1.3 UV Spectrometry

UV Spectrometry is a standard analytical method used to check the purity of DNA in the sample. The purity of DNA might be varied after extracting the genomic DNA from mycobacterium and it can be measured at an absorbance of 260nm. The DNA concentration should be in the range of ≥ 1.8 .

4.4.1.4 Ethanol Precipitation

500µl of culture sample was mixed by adding an equal volume of ethanol (100%) and placing the solutions at -20°C for 10mins. The suspensions were centrifuged for 10mins at 4000rpm for DNA precipitation. 500µl of ethanol (70%) was added to dissolve the pellet in the microcentrifuge tube and centrifuged the solution again with the above-mentioned value. Ethanol is used to eliminate the layer that surrounds the DNA and helped DNA to precipitate in the solution as the pellet forms. The pellet was collected and left to air dry. 50µl of double sterilized distilled water was added to the pellet for dissolving. The concentrated DNA was precipitated by the ethanol precipitation method and observed the DNA bands using gel electrophoresis.

4.4.1.5 Genomic DNA Purification and Precipitation

The protocol was followed as previously described in the (). 100µl of 10% SDS and 10µl of proteinase K were added to a 1ml culture tube and mixed by inverting it a few times. SDS is used to break the structure of protein and separate it from nucleic acids in the extraction process. Proteinase K is an enzyme involved to digest the protein in the cell membrane. Incubated the culture for 3hrs at 55°C and followed by centrifugation for 30mins after adding phenol(25): chloroform(24): isoamyl alcohol(1) in an equal volume to extract the contaminating protein in the solutions. The aqueous layer was observed and transferred to a sterile microcentrifuge tube. Chloroform(24): Isoamyl alcohol(1) was added to remove the phenol and supernatant was collected after centrifugation.

70µl of 3M sodium acetate and an equal volume of isopropanol was added to the supernatant to precipitate the DNA. Centrifuged the sample at 8000rpm and the pellet was washed using 70% cold ethanol. The sample was allowed to centrifuge again and the pellet was collected and left to air dry. The genomic DNA pellet was dissolved in 50µl of TE buffer solution. RNA contamination was removed by adding 1µl of RNase in the final and observing the DNA band using gel electrophoresis.

4.4.1.6 Agarose Gel Electrophoresis

The extracted DNA was visualized by agarose gel electrophoresis. 0.8% agar was suspended in 30ml of 1XTAE buffer and boiled it to completely dissolved. 1µl of ethidium bromide was added to prepared agar solutions. Ethidium bromide binds with DNA base pairs and exhibits fluorescence color when exposed to ultraviolet light which will be useful in electrophoresis for nucleic acid band identification. 6X gel loading dye was added to the sample before being placed in the well caused it contained bromophenol blue helped to observe the sample migration during electrophoresis.

4.4.2 Sequencing

4.4.2.1 16S rRNA Sequencing

It is the gold standard technique using the 16S rRNA gene to detect bacterial strains. The 16S rRNA gene is extremely conserved during evolution and contains hypervariable sections that are used to identify bacteria at the genus and species levels by recognizing their signature sequences. DNA was amplified with universal 16S rRNA primer by PCR method after DNA was isolated from the bacterial culture. The amplified DNA product was sequenced by 16S rRNA sequencing.

4.5 Analysis and Tests

4.5.1 Drug Susceptibility Tests

An agar disk diffusion assay is an easy, cost-effective, and constant method used for evaluating the effectiveness of bacteria to the drug. This assay was performed by spreading the culture evenly in a fresh MB 7H11 media plate and placing the antibiotic disk over the surface of the plate. The Petri plate containing both culture and drug components was covered with parafilm and incubated at 30°C and 37°C.

Chapter 5

RESULTS AND DISCUSSION

5.1 Mycobacterium isolation from aquatic sources

The following table represents the samples that were collected from three different locations. The collected samples were transferred into a sterile container and kept in the cold room.

S.NO	LOCATION	SAMPLES	FIGURES
1.	Kovalam	Water sample	
		Soil sample	
2.	Kasimedu	Water sample	
		Soil sample	

3.	Goa	Water sample	
		Soil sample	

Table 5.1.1: Collection of water and sand samples from various aquatic environments

5.2 Isolation

The Selective water and sand samples were inoculated into specific media MB 7H11 agar. The inoculated plates were incubated at different temperatures (30°C & 37°C). The colony's morphology and growth rate were observed and mentioned in the table



(a)



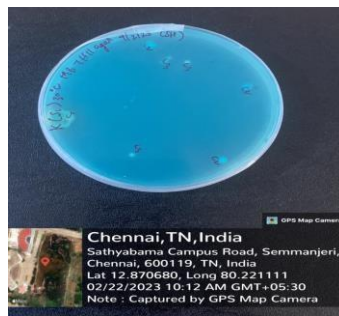
(a1)



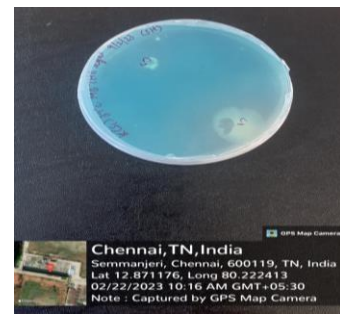
(a2)



(b)



(b1)



(b2)



(c)



(c1)



(c2)



(d)



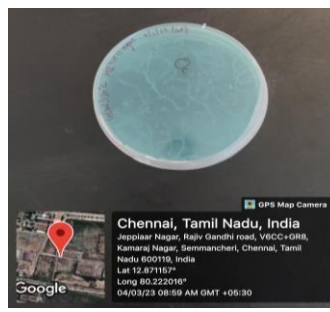
(d1)



(d2)



(e)



(e1)



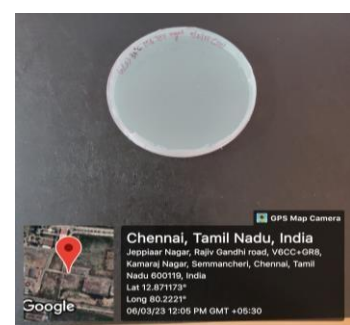
(e2)



(f)



(f1)



(f2)

- (a) - Water sample collected from Kovalam beach
- (b) - Sand sample collected from Kovalam beach
- (c) - Water sample collected from Kaasimedu beach
- (d) - Sand sample collected from Kaasimedu beach
- (e) - Water sample collected from Goa beach
- (f) - Sand sample collected from Goa beach
- (a1) (b1) (c1) (d1) (e1) (f1) - Sample incubated at 30°C
- (a2) (b2) (c2) (d2) (e2) (f2) - Sample incubated at 37°C

Figure 5.2.1: Inoculation and Incubation of sample at different temperatures

5.3 Subculture

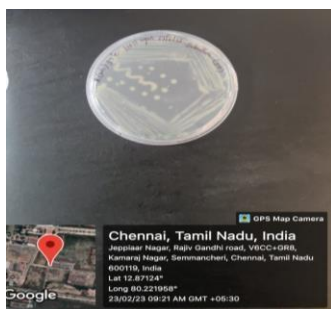
A total of 12 colonies were isolated from both samples collected from Kovalam, Kaasimedu, and Goa Beach. Each colony was quadrantally streaked on the MB 7H11 agar plate and also inoculated into MB 7H9 liquid media. The grown plates and test tubes were kept at 4°C for further testing and staining process.



(a1.1)



(a1.1.1)



(a2.1)



(a2.1.1)



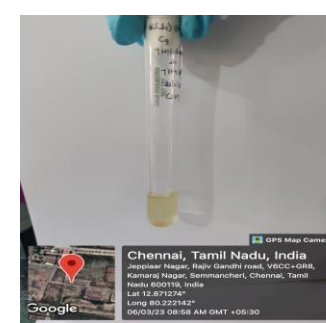
(b1.1)



(b1.1.1)



(b1.2)



(b1.2.1)



(b2.1)



(b2.1.1)



(b2.2)



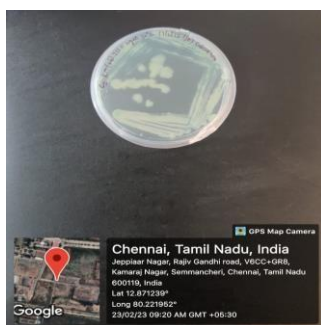
(b2.2.1)



(c1.1)



(c1.1.1)



(c1.2)



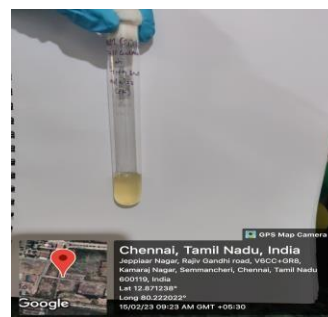
(c1.2.1)



(d1.1)



(d1.1.1)



(d2.1)

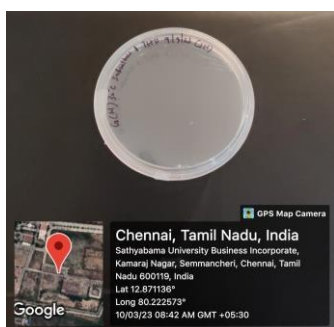


(d2.1.1)



(d2.2)

(d2.2.1)



(e1.1)



(e1.1.1)

Figure 5.3.1: Pure culture isolation from Middlebrook 7H11 agar plate and Middlebrook 7H9 liquid media.

5.4 Colony differentiation

The isolated colonies were observed in four different colors pale yellow, White, Creamy white, and transparent white. The Morphology of each colony was examined by the naked eye. The colonies and their differentiation were represented in the following table.

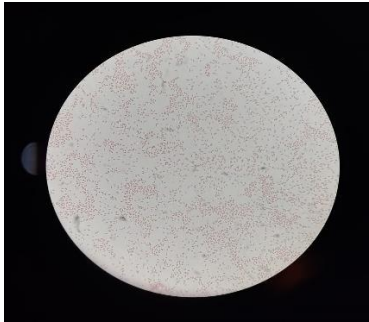
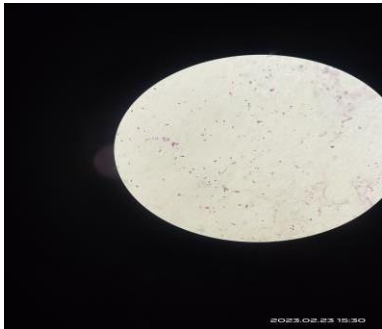
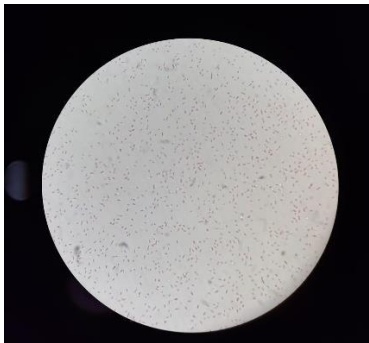
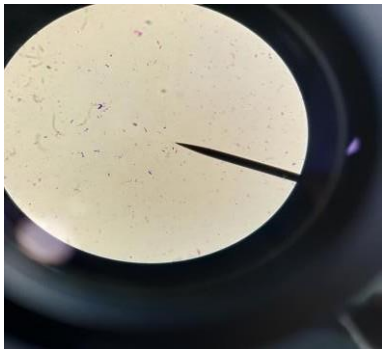
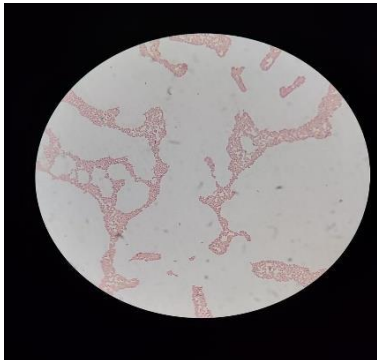
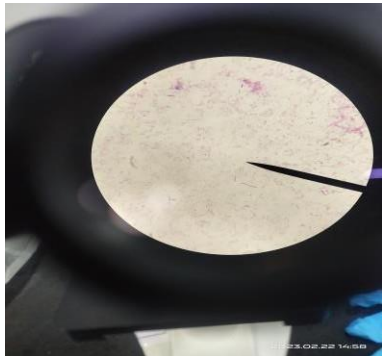
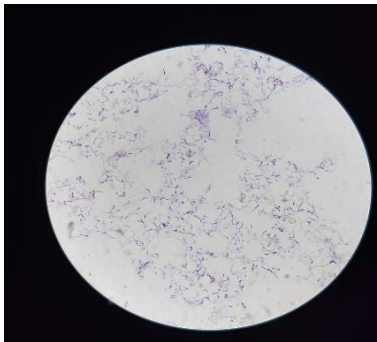
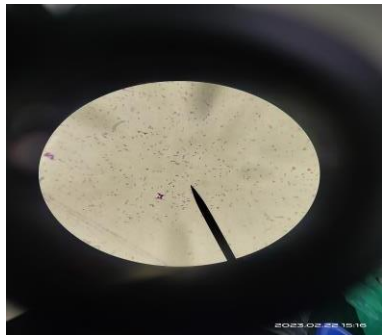
SOURCES	SAMPLES	INCUBATION TEMPERATURE	OBSERVED COLONIES	GROWTH	MORPHOLOGY	PIGMENT PRODUCED
Kovalam	Water sample	30°C	Colony 1	4 day – Rapid growth	Moist Smooth	Pale yellow
		37°C	Colony 1	4 day – Rapid growth	Moist Smooth	Creamy white
Kovalam	Sand sample	30°C	Colony 1	5 day – Rapid growth	Moist Smooth	White
			Colony 2	5 day – Rapid growth	Dry Smooth	Transparent white
		37°C	Colony 1	5 day – Rapid growth	Moist Smooth	Transparent white
			Colony 2	5 day – Rapid growth	Dry Smooth	Transparent white
Kaasimedu	Water sample	30°C	Colony 1	5 day – Rapid growth	Dry Rough	Pale yellow

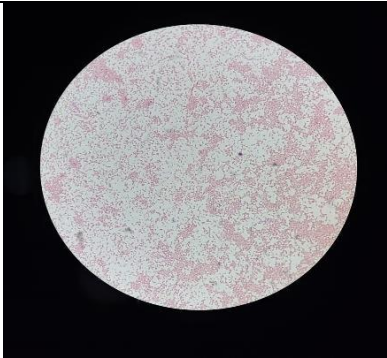
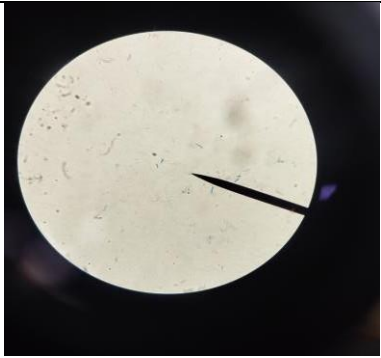
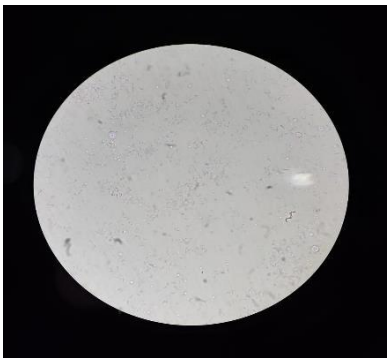
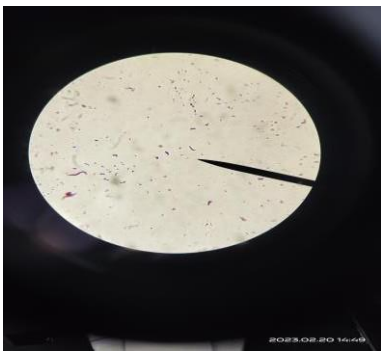
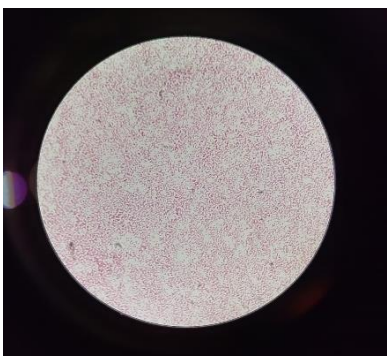
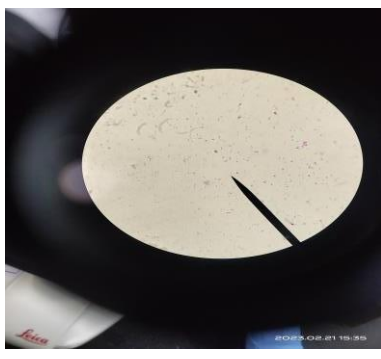
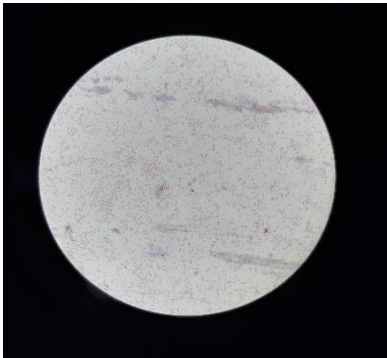
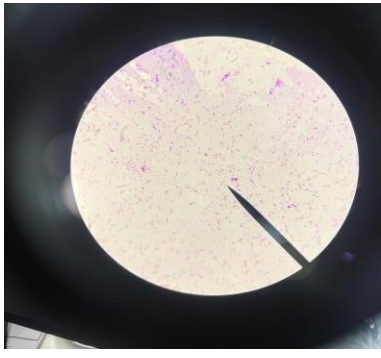
			Colony 2	5 day – Rapid growth	Dry Rough	Pale yellow
		37°C	No growth	-	-	-
Kaasimedu	Sand sample	30°C	Colony 1	1 day – Rapid growth	Moist Smooth	White
		37°C	Colony 1	1 day – Rapid growth	Moist Smooth	Creamy white
			Colony 2	1 day – Rapid growth	Moist Smooth	Creamy white
Goa	Water sample	30°C	Colony 1	20-day – Slow growth	Moist Smooth	White
		37°C	No growth	-	-	-
Goa	Sand sample	30°C	No growth	-	-	-
		37°C	No growth	-	-	-

Table 5.4.1: Differentiating the colonies based on their morphology, growth rate, and pigment production.

5.5 Staining

A staining technique was performed for the detection of mycobacterium. Ten colonies were shown pink color and indicated as gram-negative bacteria. The remaining two colonies were exhibited purple color and denoted as gram-positive bacteria in the gram staining method. In Ziehl neelsen staining, ten cultures were confirmed as mycobacterium by showing red rod bacilli. The other two cultures were identified as non-acid fast organisms due to their blue color appearance under microscopic observation. The staining results were represented with figures in the following table. Only two cultures were chosen out of twelve cultures that had shown positive results in both gram stain and acid-fast stain and they were processed for further examination.

S.NO	ISOLATED COLONIES	GRAM STAIN	ACID-FAST STAIN
1.	(a1.1)		
2.	(a2.1)		
3.	(b1.1)		
4.	(b1.2)		
5.	(b2.1)		

			
6.	(b2.2)		
7.	(c1.1)		
8.	(c1.2)		

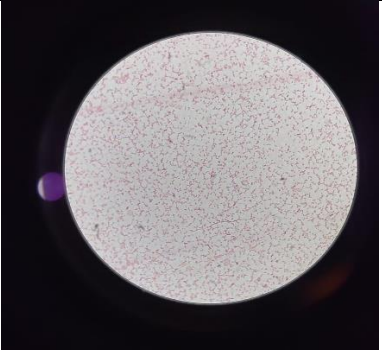
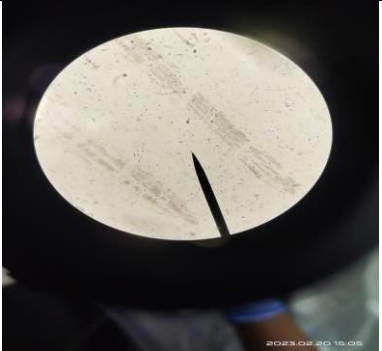
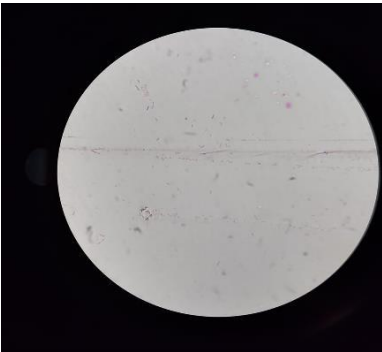
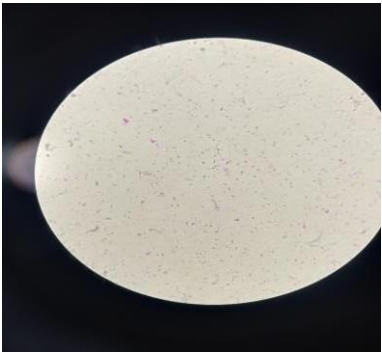
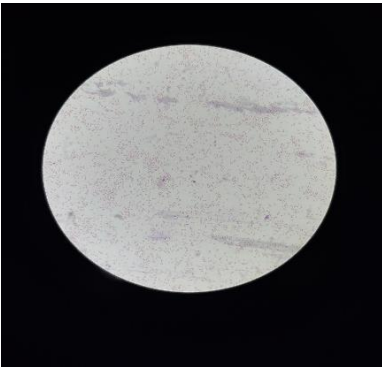
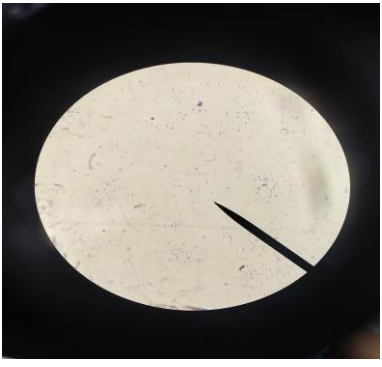
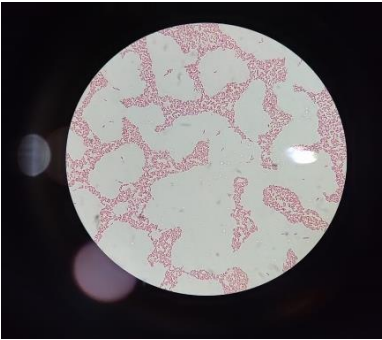
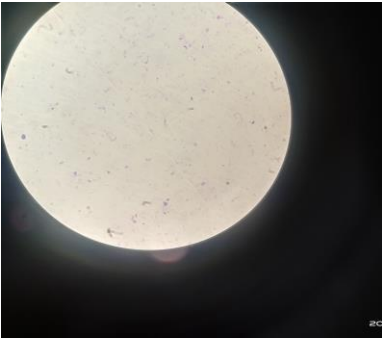
9.	(d1.1)		
10.	(d2.1)		
11.	(d2.2)		
12.	(e1.1)		

Table 5.5.1: Gram stain and Acid-fast staining images of the culture

5.6 Motility

Three colonies were selected to check the motility of organisms. A single colony of each plate was scrapped from the surface of the Middlebrook 7H11 agar medium with a disposable loop. The colony was inoculated into a Sterile test tube that contain freshly prepared 5ml Middlebrook 7H9 broth and incubated for 24hrs at 30°C and 37°C. Culture Turbidity was observed by the naked eye. A drop of culture was placed in the center of the coverslip and the corner of the coverslip was applied with Vaseline. The concave glass slide was used to cover the entire coverslip to check the motility. Bacterial motility was recognized using a light microscope at 40X magnification.

S.NO	CULTURE	OBSERVANCE
1.	(c1.2)	Motile
2.	(b1.2)	Non-motile
3.	(b2.2)	Non-motile

Table 5.6.1: Observation of bacterial motility by hanging drop method

5.7 Biochemical assay

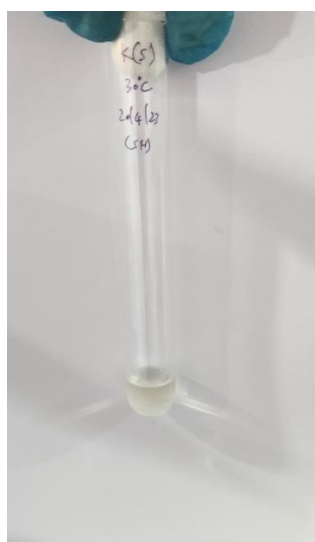
(c1.2), (b1.2), and (b2.2) are the colonies selected for tween 80 hydrolysis and urease test. In tween 80 hydrolysis was used to distinguish pathogenic and non-pathogenic species. All three colonies were shown amber colored complex in tween 80 indicating that organisms were not able to hydrolyze the tween 80 substrate. Urease tests are used to evaluate the organisms which able to convert urea into ammonia and carbon-dioxide results pink color formation. Colonies that were tested showed moderate pink color after 6 days of incubation and indicated positive results in the urease test.



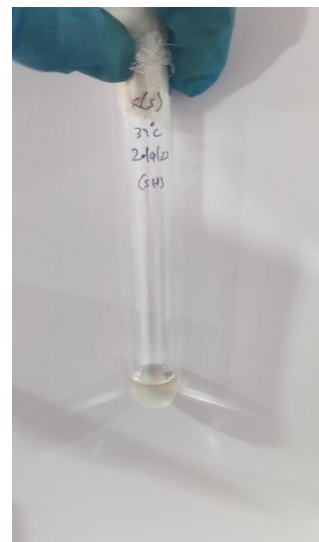
(Control)



(c1.2)



(b1.2)



(b2.2)

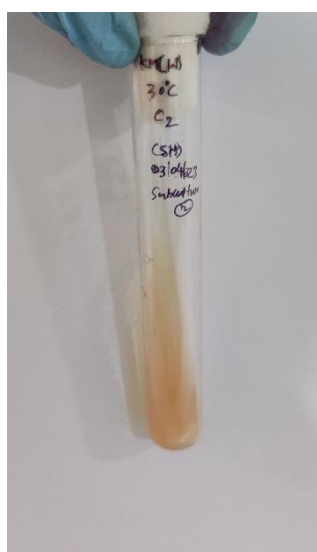
Figure 5.7.1: Identification of bacteria using Tween 80 Hydrolysis

DAYS OF INCUBATION	COLOUR OBSERVATION		
	(c1.2)	(b1.2)	(b2.2)
Day 1	Negative	Negative	Negative
Day 5	Negative	Negative	Negative
Day 12	Negative	Negative	Negative

Table 5.7.1: Bacterial color observation in tween 80 hydrolysis on various days of incubation.



(Control)



(c1.2)



(b1.2)



(b2.2)

Figure 5.7.2: Identification of bacteria using Urease test

DAYS OF INCUBATION	COLOUR OBSERVATION		
	(c1.2)	(b1.2)	(b2.2)
6 hrs	Negative	Negative	Negative
Day 1	Negative	Negative	Negative
Day 2	Negative	Negative	Negative
Day 3	Negative	Negative	Negative
Day 4	Negative	Moderately positive	Moderately positive

Day 5	Negative	Moderately positive	Moderately positive
Day 6	Moderately positive	Moderately positive	Moderately positive

Table 5.7.2: Bacterial color observation in urease test on various days of incubation.

5.8 Sequencing

The DNA was extracted from culture (c1.2) by thermolysis method. 8µl of extracted DNA was mixed with 2µl of loading dye and allowed to run in 0.8% agarose gel electrophoresis and the DNA band was clearly visualized under an ultraviolet transilluminator. The resultant band was compared with a 5µl of DNA marker which was found to be more than 10,000bp. The isolated culture (c1.2), (b1.2), and (b2.2) were sequenced by 16S rRNA sequencing, and the distant trees were collected from NCBI for all three cultures mentioned below.

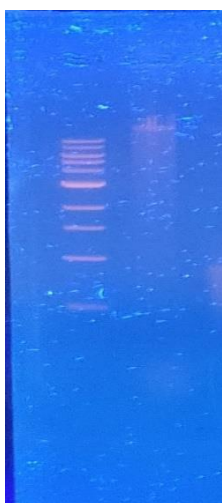


Figure 5.8.1: This figure represents the DNA band observed by agarose gel electrophoresis

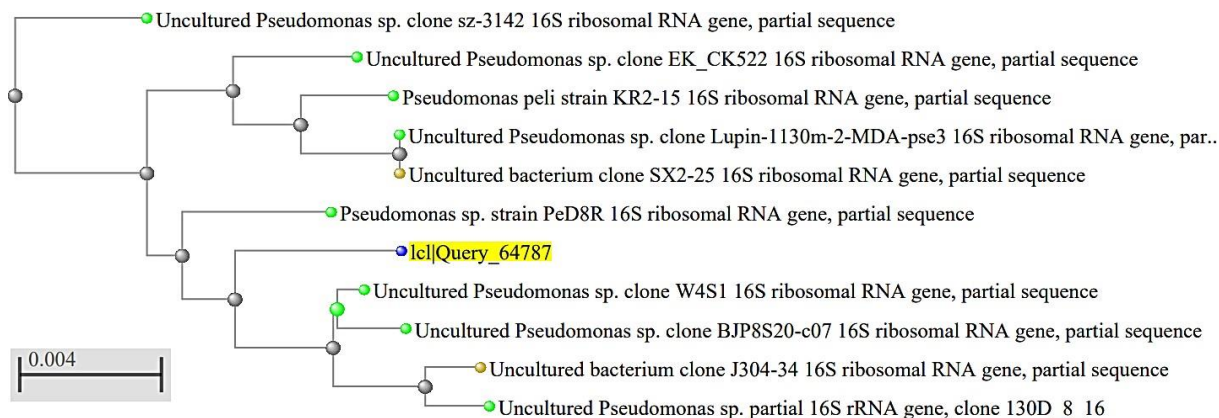


Figure 5.8.2: Distant tree from NCBI showed (c1.2) colony as *Pseudomonas peli*

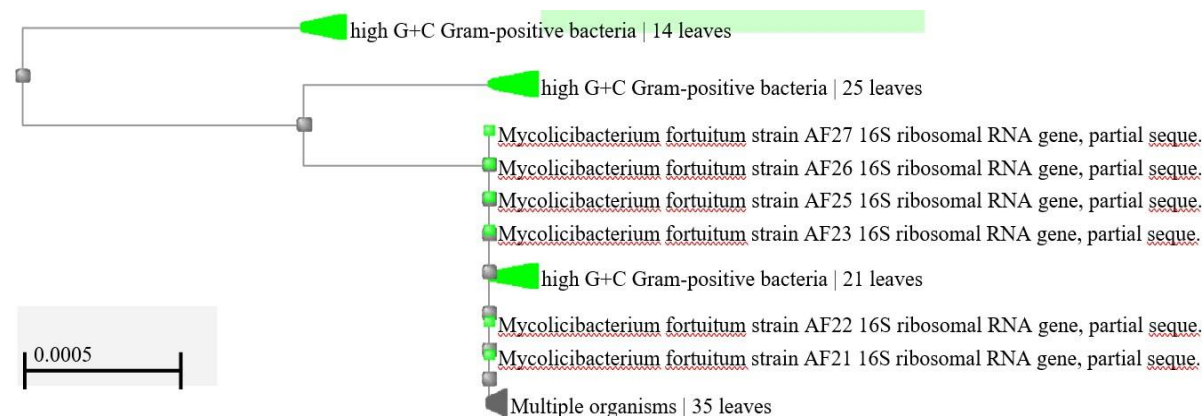


Figure 5.8.3: Distant tree from NCBI showed (b1.2) colony as *Mycobacterium fortuitum*

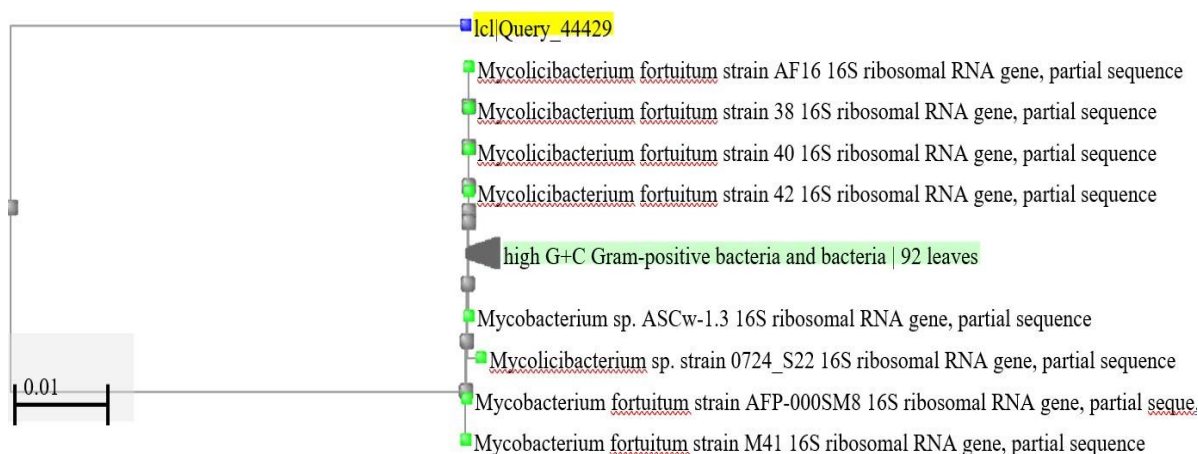
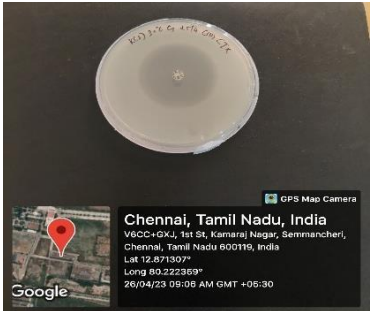


Figure 5.8.4: Distant tree from NCBI showed (b2.2) colony as Mycobacterium

5.9 Drug susceptibility testing

The species which had been confirmed as mycobacterium by sequencing method were selected for the drug susceptibility test. The antimicrobial disc was placed in culture plates and the zone of inhibition was observed after incubating at different temperatures. The used antibiotics and the activity of bacteria were mentioned in the below table.

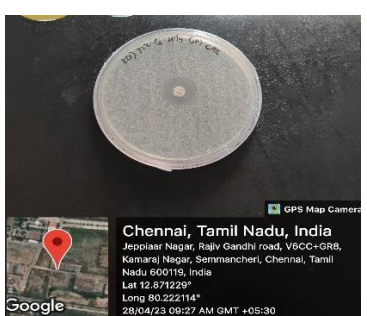
S.NO	ANTIMICROBIAL COMPOUND	DISK CONTENT	(b1.2) SENSITIVE/ RESISTANCE	ZONE OF INHIBITION (cm)	RESULTS
1.	Control	-	-	-	 A photograph of a white antibiotic disk on a dark surface. Below the disk is a Google Map Camera overlay showing the location in Chennai, Tamil Nadu, India, with coordinates Lat 12.87160° and Long 80.22203°.
2.	Amikacin (AMK)	30mcg	Sensitive	3.2 cm	 A photograph of a white antibiotic disk on a dark surface. Below the disk is a Google Map Camera overlay showing the location in Chennai, Tamil Nadu, India, with coordinates Lat 12.87125° and Long 80.22273°.
3.	Amoxicillin (AMC)	30mcg	Sensitive	1cm	 A photograph of a white antibiotic disk on a dark surface. Below the disk is a Google Map Camera overlay showing the location in Chennai, Tamil Nadu, India, with coordinates Lat 12.87148° and Long 80.22279°.
4.	Aztreonam (ATM)	30mcg	Sensitive	2.6cm	 A photograph of a white antibiotic disk on a dark surface. Below the disk is a Google Map Camera overlay showing the location in Chennai, Tamil Nadu, India, with coordinates Lat 12.87182° and Long 80.22216°.
5.	Cefoxitin (FOX)	30mcg	Sensitive	2.3cm	

					
6.	Cefotaxime (CTX)	30mcg	Sensitive	3.4cm	
7.	Ceftazidime (CAZ)	30mcg	Sensitive	2.6cm	
8.	Gentamicin (GEN)	10mcg	Sensitive	2.8cm	
9.	Piperacillin / Tazobactam (TZP)	100/10mcg	Sensitive	3.9cm	

10.	Tetracycline (TCY)	30mcg	Sensitive	1.8cm	
11.	Trimethoprim (SXT)	5mcg	Sensitive	3.4cm	

Table 5.9.1: Drug susceptibility test of *Mycobacterium fortuitum* (b1.2).

S.NO	ANTIMICROBIAL COMPOUND	DISK CONTENT	(b2.2) SENSITIVE/ RESISTANCE	ZONE OF INHIBITION (cm)	RESULTS
1.	Control	-	-	-	
2.	Amikacin (AMK)	30mcg	Sensitive	5.5cm	
3.	Amoxicillin (AMC)	30mcg	Sensitive	2cm	

					
4.	Aztreonam (ATM)	30mcg	Resistance	-	
5.	Cefoxitin (FOX)	30mcg	Sensitive	3.5cm	
6.	Cefotaxime (CTX)	30mcg	Resistance	-	
7.	Ceftazidime (CAZ)	30mcg	Sensitive	1.5cm	

8.	Gentamicin (GEN)	10mcg	Sensitive	3.3cm	
9.	Piperacillin / Tazobactam (TZP)	100/10mcg	Sensitive	2cm	
10.	Tetracycline (TCY)	30mcg	Sensitive	2cm	
11.	Trimethoprim (SXT)	5mcg	Sensitive	4cm	

Table 5.9.2: Drug susceptibility test of *Mycobacterium* (b2.2).

Chapter 6

CONCLUSION

Various studies have demonstrated the clinical importance of additional mycobacteria, despite that research has mostly been concentrated on the species of *Mycobacterium ulcerans*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae*. During these past three decades, other *Mycobacterium* species outside *M. Tuberculosis* becomes more widely acknowledged as pathogens, because advanced microbiological techniques are being used more frequently. Numerous nontuberculous species seem widespread and can be found in milk, other foods, water, soil, and domestic as well as wild animals.

Water is the primary transmission route for cutaneous tropism mycobacteria. NTM is constantly in touch with people through the water. Especially healthy individuals who come into exposure to water frequently, whether through their line of work or leisure pursuits. There are a few basic sense precautions that should be mentioned such as disinfecting wounds after an accident and keeping them dry. Keeping a chlorine level at 0.8mg/ml in swimming pools prevents mycobacteria from multiplying. Additionally, UV germicide lamps can effectively purify aquarium water for mycobacteria provided that they are utilized in hygienic settings with proper flow rate. NTM can be found in the tap water utilized to make reactive chemicals in hospitals and to clean hemodialyzers. Once within the device, mycobacteria grow and are challenging to get rid of (Dailloux et al., 1999). Although they cannot be completely eradicated, there are some steps that can be taken to reduce the mycobacterial populations. About 25 different nontuberculous mycobacterial species have been linked to human disease. Such infections typically have severe granulomas, oedema, ulceration, and cavitation along with a slow growth rate. However, immune-suppressed individuals, especially those who have acquired immune deficiency syndrome frequently get mycobacterial diseases.

According to some authors, antimicrobial drugs only had positive benefits (Chow et al., 1987). Nevertheless, the most of research advises surgical treatment and two antibiotics for two to six months (Plaus & Hermann, 1991). Localized infections in immunocompromised patients were successfully cured with just surgery in one series (Marchevsky et al., 1985). Environmental mycobacteria could infect or potentially harm

people under favorable circumstances, however, it also has been observed that *M. avium* can sicken people without favorable situations (Prince et al., 1989). Even though the disease caused by nontuberculous mycobacterium is uncommon, it is crucial to take this curable cause into account when making a clinical assessment for inflammation and pain in the extremities. First-line antituberculosis drugs frequently fail to kill rapidly proliferating mycobacteria, In contrast, conventional antibiotics drugs successfully kill them.

Ciprofloxacin, monocycline, and clarithromycin are among the antibiotics that are most efficient and effective. substantial research is currently being done on *Mycobacterium indicus pranii* as an immunomodulating agent against a variety of different human disorders. Also, it has recently been found to exhibit more putative *M. leprae* and *M. tuberculosis* antigens (Sharma et al., 2017; Faujdar et al., 2011). The ability to elicit a powerful Th1 response, increased production of pro-inflammatory cytokines, stimulation of APCs as well as lymphocytes, and induction of polyfunctional T cells that are specific to *M. tuberculosis* are among MIP's possible immunomodulatory features that have been reported in studies. During *M. tb* infection, each of these plays a vital role in the host immunological response (Singh et al., 2019). Its commercial name, Immuvac refers to its clinical application in the treatment of leprosy intervention. The use of interferon- γ in nontuberculous mycobacterium infections has just been revealed (Holland et al., 1994). In this work, *Mycobacterium* was isolated from aquatic sources such as water and sand samples and determined their antibiotic pattern. The future work of this study is to compare the protein stability of culture with isolates collected from clinical samples by an in-silico method that will be helpful for future therapeutic development.

Chapter 7

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