PREVALENCE OF NON-TUBERCULOUS MYCOBACTERIA IN AQUATIC ENVIRONS

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Thesis submitted to the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar in partial fulfillment of the requirement of the

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Dedicated with

Love and Respect

To

My Dear Parents and Teachers

DEPARTMENT OF FISHERY MICROBIOLOGY
KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR

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CERTIFICATE

This is to certify that the thesis entitled **"PREVALENCE OF NON-TUBERCULOUS MYCOBACTERIA IN AQUATIC ENVIRONS "** submitted by **Mr. Kiran Kumar.B.S** for the award of Master of Fisheries Science in Fishery Microbiology of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of research done by him during the period of study in the University under my supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship or other similar titles.

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Kiran Kumar, B. S.,

CONTENTS

Page No.

1. INTRODUCTION

Tuberculosis (TB) and leprosy have afflicted humankind from times immemorial. Approximately one third of the world's population is currently infected with tuberculosis caused by *Mycobacterium tuberculosis* and nearly two million people die of TB annually (Josephine *et al.,* 2003). TB continues to cause a devastating impact on the world's population (Dubos and Dubos, 1952; Collins, 1982; Daniel, 1994). The World Health Organization has estimated that one third of the world's population is infected with TB, with approximately 8 million new cases diagnosed annually (Dye *et al.*, 1999). In the early history of humankind, TB was probably sporadic, but as population densities increased, the disease began to spread (Daniel, 1994) and reached epidemic proportions in Western Europe during the 17th, 18th and19th centuries (Dubos and Dubos, 1952; Daniel, 1994; Cole, 2002). As a consequence of the industrial revolution, with the dispersal of people many countries changed from primarily agrarian to urban communities. Coincident with these changes were overcrowded living conditions, poor sanitation and malnutrition, all of which enhanced both the spread of *M. tuberculosis* and the susceptibility of the population to disease (Dubos and Dubos, 1952; Daniel, 1994; Cole, 2002). Many of the new tuberculosis cases result atleast in part from the elevated incidence of tuberculosis in acquired immuno deficiency syndrome (AIDS) patients. After influenza and pneumonia, tuberculosis is the leading cause of death by an infectious agent.

The genus *Mycobacterium* contains a number of obligate and opportunistic pathogens that affect humans and animals alike. Among the obligate pathogens, the causative agent of tuberculosis, *M. tuberculosis* and leprosy, *M. leprae* are the important ones. Several environmental mycobacteria such as *M. simiae, M. kansasii* and *M. haemophilum* cause opportunistic infections among immuno-compromised patients and are generally referred to as atypical mycobacteria or non-tuberculous mycobacteria. The importance of non-tuberculous mycobacteria in human pathology has and is being increasingly recognized due to the emergence of AIDS (WHO, 2002).

Atypical mycobacteria survive chlorination in municipal water and use it as vector to get to humans. Wide spread chlorination of water has likely selected more resistant environmental mycobacteria species and potentially explains the shift from *M. scrofulaceum* to *M. avium* as a cause of cervical lymphadenitis in children. Mycobacteria are unique as they are resistant to attack by the killing mechanisms of macrophages of the hosts immune system and are in fact able to multiply inside the macrophages. Thus the bacilli flourish for years inside the host prior to causing disease and at times killing the host. They also have the ability to survive long periods of starvation or aridity without dehydration (Todd *et al.,* 2004).

Natural aquatic environment is a recognized source of mycobacteria and are known to cause diseases in both humans and fish. These mycobacterial infections are quite common in fish from fresh and marine water body and are reported from temperate as well as tropical environments. The major etiological agents for fish mycobacteriosis are *M. chelonae, M. fortuitum* and *M. marinum.* These produce disease conditions of chronic to subacute in nature in fish with symptoms similar to human tuberculosis. This made their earlier recognition be labelled as "piscine tuberculosis". Apart from causing disease in fish, mycobacteria are also implicated in "fish handlers' disease" in people who have cuts and wounds and involved in the handling of fish (Belas *et al.*, 1995). Though fish mycobacteriosis is not the same as human tuberculosis, it is one of the most common disease affecting aquarium fish. *M. marinum* and *M. fortuitum* are zoonotic causing skin infection and hypersensitivity in aquarists (Swift and Cohen, 1962). Many reports suggest the potential role of fish in the transmission of the disease 'Buruli ulcer' caused by *M. ulcerans* (Eddyani *et al.*, 2004).

Against this background, the present study was taken up to look at the prevalence of non-tuberculous mycobacteria associated with the aquatic environment, with the following objectives:

1. Isolation of *Mycobacterium* species from water and sediment samples collected from sea, estuaries, rivers, shrimp farms, fishponds and aquaria.
2. Rapid detection of *Mycobacterium* species using molecular techniques.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2.1. History

Mycobacterial infections in fish are generally referred to as tuberculosis or mycobacteriosis, irrespective of the specific identity of the causal organism. The disease is largely chronic to subacute in nature and affects fish in fresh water, brackish water and marine water.

Historical descriptions of acid-fast bacteria cultured from tuberculosis-like infections in fish and reptiles were reported 14 years before Koch cultured the tubercle bacillus (Villemin *et al.,* 1868). The first report of a mycobacterial infection in fish has been attributed to Bataillon *et al.* (1897) who isolated acid-fast bacilli from a tuberculous lesion of a common carp (*Cyprinus carpio*) and named the isolate as *Mycobacterium piscium* on the basis of its derivation (Bataillon *et al.,* 1902).

Aronson (1926) had published a report characterizing *Mycobacterium marinum* as the bacterium responsible for fish death in an aquarium. Baker and Hagan (1942) described that *M. marinum* was one of the principal mycobacteria responsible for fish mycobacteriosis and they also isolated it from platy. Ross (1960) isolated mycobacteria from upstream migrant and hatchery held salmon. Mycobacteriosis of Pacific salmonids was first identified by Earp *et al.* (1953). The second fish mycobacterial pathogen to be recognised and isolated was from neon tetra fish (*Hyphessobrycon innesi*) by Nigrelli (1953) and later identified by Ross and Brancato (1959) as *M. fortuitum. M. fortuitum* originally described as a pathogen of gias (giant frogs of Brazil) by Darzins (1950).

M. chelonei was first isolated by Friedmann (1903) from a turtle in marine aquarium and Ashburner (1977) first reported an incident of *M. chelonei* infection in Chinook salmon (*Onchorynchus tschawytscha*) in Australia. *M. chelonei* has now been linguistically corrected to *M. chelonae* (Wayne and Kubica, 1986).

A common and serious bacterial disease of fish is mycobacteriosis, most often caused by one of the three following gram-positive, acid-fast bacilli, *M. marinum*, *M. fortuitum*, and *M. chelonae*. Mycobacteriosis is a chronic progressive disease, causing systemic granulomas in any tissue or internal organ and often leading to death (Austin and Austin, 1993; Smith, 1997). Mycobacteriosis is a common disease in wild captive fish (Chinabut, 1999) and ornamental marine and fresh water fish (Noga, 2000). *M. marinum, M. chelonae, M. abscessus, M. fortuitum, M. peregrinum* and *M. haemophilum* have been found associated with mycobacteriosis in Zebrafish (Astrofsky *et al.,* 2000).

Massive mortalities due to mycobacteriosis have been reported among fish grown in intensive aquaculture systems. Mycobacteriosis was first documented as disease of cultured fish in the 1950's when mortalities among hatchery raised *Onchorynchus* and *Salmo* spp. increased dramatically and morbidity even reached 100% in some populations (Wood and Ordal, 1958). Among other fish that are affected by mycobacteriosis are striped bass, *Morone saxatilis* (Aronson, 1926), sea bass, *Dicentrarchus labrax* (Colorni, 1992), tilapia, *Oreochromis mossambicus* (Noga *et al.,* 1990), summer flounder, *Paralichthys dentatus* (Hughes *et al.,* 2002), plaice, *Pleuronectus platessa* (Timur *et al.,* 1977), yellow perch, *Perca flavescens* (Daoust *et al.,* 1989), snakehead, *Channa striatus* (Chinabut *et al.,* 1990) and pejerrey, *Odonthestes bonariensis* (Hatai *et al.,* 1993). Fish cultured for the ornamental pet trade are also susceptible, with guppy, *Labistes reticulatus* (Bragg *et al.,* 1990), tetra, *Moenkhausi sanctaefilomenae* (Shamsudin *et al.,* 1990), angelfish, *Pterophyllum spp* (Smith, 1997), and gold fish, *Carassius auratus (*Talaat *et al.,* 1999) having been reported with mycobacteriosis.

2.2. Zoonosis

Mycobacterium spp. in addition to causing morbidity and mortality in aquarium fish, have documented zoonotic potential for human, especially in immunosuppressed individuals (Swift and Cohen, 1962; Wilson, 1976). Infections in humans of any of these aquatic species of *Mycobacterium* generally produce localized cutaneous granulomas commonly called 'Fish handlers' disease' or 'Swimming pool granulomas'. These localized papulopustular lesions are generally found on the hands and arms and often require long-term medical therapy for mycobacteria are sometimes isolated from the lungs, lymph nodes and other internal organs (Inglis *et al.,* 1993). An outbreak of mycobacteriosis in an aquarium thus can be a serious zoonotic problem for the owners of the infected aquarium systems.

Majority of fresh water tropical fish are captive bred in many types of culture facilities throughout the world but most marine fish species are still acquired directly from the wild. This is significant because fish derived from the wild environment are generally more susceptible to the stress of the captive environment and are more frequently predisposed to overt infections of mycobacteriosis (Wolke and Stroud, 1978). Tuberculoid infections in people using public swimming pools were reported in 1939 from Sweden and in 1955 from the US. Linell and Norden identified the causative organism in 1954 after persons who had used the same public swimming pool were diagnosed with granulomatous skin (Smith, 1997; Dobos *et al.,* 1999). Initially, they called the pathogen *M. balnei* which subsequently appeared to be synonymous with *M. marinum* and the disease caused was called "swimming pool granuloma". The names 'fish tank granuloma' and 'fish handlers disease' are sometimes used because of the associations with home aquarium and water related activities such as fishing, swimming and boating (Ang *et al.,* 2000). As with mycobacterial disease in poikilothermic organisms, *M. marinum* infections in mammals persist because the bacteria can survive even in homeothermic host cells. *M. marinum* invades and proliferates in fibroblasts, epithelial cells and macrophages (Mor, 1985; Ramakrishnan and Falkow, 1994).

Disease caused by *M. marinum* infection has been well documented since 1970's and human tuberculous skin lesions described between 1939 and 1954 were eventually attributed to *M. marinum* infection (Collins *et al.,* 1985). Human *Mycobacterium* spp. infection has been increased in both immuno-competent and immuno-compromised individuals in the last decade, even though the disease is most likely under-reported and often misdiagnosed (Dobos *et al.,* 1999). Human atypical *Mycobacterium* infections are often associated with direct handling of infected fish or with exposure to water, especially in swimming pools, saunas and aquaria.

Occasionally aquatic mycobacteria can spread to internal human body systems and have been isolated from pulmonary lesions (Chinabut, 1999), synovial fluid and muscles (Blacklock and Dawson, 1979). A number of cases of *M. marinum* infection have been documented among HIV-infected people and these include disseminated infections and infections resistant to standard anti-tuberculosis treatments (Glaser *et al.,* 1994). The bacteria can gain entrance into the human body through traumatic lesions on the skin and affected hosts. Infections are usually limited because the optimal growth temperature for these bacteria is 25-35°C and growth appears to be inhibited at 37°C (El-Etr *et al.,* 2001).

M. marinum infection may be an occupational hazard for certain professionals like pet shop workers as well as in fish fanciers who keep an aquarium at home, causing characteristic "fish fancier finger syndrome" (Wheeler and Graham, 1989). Although infection may be caused by direct injury from the fish fins or bites, most are acquired during the handling of the aquaria such as cleaning or changing the water (Bhatty *et al.,* 2000; Jernigan and Farr, 2000). Indirect infection has also been described due to a child's bath that was used to clean out a fish tank. Although less common than *M. marinum,* two of the mycobacterial species capable of infecting humans are *M. fortuitum* and *M. chelonae* (Zenone *et al.,* 1999).

2.3. Habitat

Non-tuberculous mycobacteria are relatively common environmental bacteria. This means that they can live in the aquatic environment and do not require the presence of fish or fish tissue to survive. They have been isolated from many natural habitats, drinking water supplies, swimming pools, coastal waters and aquaculture facilities (Landsdell *et al.,* 1993).

Bacteria of the genus *Mycobacterium* are not rare in the environment and have been isolated from natural habitats (Kamala *et al*., 1994). These acid-fast bacteria could be found in both freshwater and marine ecosystems (Viallier *et al.,* 1967; Eilertsen, 1969; Showalter *et al.,* 1974; Falkinham *et al.,* 1978; Wendt *et al.,* 1980). They were first isolated from aquatic animals and later from the water in which the animals lived (Collins *et al*., 1984). There were many reports that mycobacteria could survive in seawater for long periods of time (Jamieson *et al.,* 1976; Falkinham *et al.,* 1978; Gruft *et al.,* 1981).

M. marinum has been isolated from many aquatic environments including fish tanks, swimming pools and hot tubs. (Baker and Hagan, 1942; Ross, 1960; Barrow and Hewitt, 1971; Landsdell *et al.,* 1993). *M. gordonae, M. kansasii, M. avium, M. avium- intracellularae, M. chelonae* and *M. fortuitum* have also been found associated with aquaria and also isolated from water (Pattyn *et al.,* 1971; Goslee *et al.,* 1976; Caroli *et al.,* 1982) Gruft *et al.* (1981) isolated *M. intracellularae* and *M. scrofulaceum* from estuarine and marine waters.

Opinions vary regarding the true habitat of free-living mycobacteria. Collins *et al.* (1984) consider them to be truly aquatic and their principal habitat more likely to be wet soil or stagnant water in soil contact. They were able to replicate in wet or flooded soil but not in dry soil because of lack of adequate moisture (Kazda, 1983). The mycobacterial microflora of a habitat multiplies in favourable temperature and humidity.

Saprophytic mycobacteria are found chiefly in natural substrates and in the environment of living beings. They have the capacity to survive and multiply over a wide range of environmental conditions. Both potentially pathogenic and saprophytic mycobacteria have been isolated from inland freshwater, aquaria, cattle water tanks, swimming pools, rain water tanks and tap water as contaminants. Colliery water, waste water, sea water, samples of a wide variety of soils, tropical vegetation, deep litter, saw dust also produced potential pathogens on culture. The occurrence of obligate pathogenic mycobacteria in nature is associated with their discharge from disease processes into natural biotopes (Kazda, 1983).

2.3.1. Water

Many of the mycobacterial organisms naturally occur in the aquatic environment. There are few reports in which mycobacteria have been isolated from water in which diseased fish live (Goslee *et al.,* 1976; Dailloux *et al.,* 1992). *Mycobacterium* has been detected in both freshwater and the marine environment. Gruft *et al.* (1975) and Jamieson *et al.* (1976) revealed that mycobacteria can survive even in sea water. Falkinham *et al.* (1978) recovered acid-fast bacilli from coastal waters off South-Eastern USA, while Viallier (1967) isolated mycobacteria from water samples from the sea. Joynson (1979) demonstrated that *M. kansasii* could survive upto 12 months in water without any change in culture characteristics.

Wayne and Sramek (1992) isolated *M. simiae* from the water supply of a hospital in Gaza. Mario *et al.* (2005) reported environmental mycobacteria such as *M. avium, M. kansasii* and *M. xenopi* frequently from drinking water and hospital water distribution systems. The presence of environmental mycobacteria in tap water has been linked to nosocomial infections and pseudoinfections. (Joynson, 1979) demonstrated that, after inoculation, *M. kansasii* survived in water up to 12 months without any change in cultural or lipid characteristics. The organism failed to survive in soil and he suggested that the natural habitat of *M. kansasii* is water.

2.3.2. Soil / Sediment

Soil or sediment is another habitat from which *Mycobacterium spp.* have been isolated. Kubica *et al.* (1963) and Pushpalatha (2004) successfully isolated *M. fortuitum, M. smegmatis* and *M. phlei* from soil samples. The presence of a variety of mycobacteria in the soil of an agricultural farm was reported by Donoghue *et al.* (1997). Katila *et al.* (1995) identified potential human pathogenic mycobacterial species in water and soil samples.

2.4. Classification and General Characteristics

Taxonomically, mycobacteria belong to the genus *Mycobacterium*, which is the single genus within the family of Mycobacteriaceae in the order Actinomycetales (Stackbrandt *et al.*, 1997). Although morphology of mycobacteria may vary from coccobacilli to long slender rod-shaped cells (0.3-0.6 μ X 0.5-6.0μ), they share a distinctive staining property which depends upon their lipid-rich cell walls. They are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, however, the mycobacteria resist decolorization with acidified organic solvents and are, therefore, called acid-fast.

The genus *Mycobacterium* is divided into two major groups of rapid and slow growers. The obligate and opportunistic mycobacterial pathogens are slow growers, having an average division time of 12 h to 24 h and requiring up to 15 to 20 days to grow on culture media. On the other hand, most of the environmental mycobacteria grow in 2 to 7 days on culture media.

Based on clinical importance, mycobacteria can be classified into the following three principal groups (Rastogi *et al.*, 2001).

1. Obligate pathogens, *M. tuberculosis*, *M. leprae* and *M. bovis*.
2. Opportunistic or potential pathogens, *M. simiae, M. avium* and *M. xenopi*.
3. Rare pathogens including saprophytes, *M. smegmatis* and *M. phlei*.

The mycobacteria were initially divided into *M. tuberculosis* complex non-tuberculosis or atypical mycobacteria, as this discrimination could be easily performed in most microbiological laboratories. For practical purposes, Runyon (1959) proposed a classification of atypical mycobacteria into four groups based on phenotypic characteristics such as pigmentation and rate of growth. The first 3 groups included only slow growers, which require more than one week to grow. They are photochromogenic (colonies acquire pigmentation in the presence of light only), non-photochromogenic (non-pigmented colonies) scotochromogenic (colonies are pigmented in the presence or absence of light). The fourth group included rapidly growing mycobacteria.

It has been suggested that photochromogenic carotenoids protect mycobacteria against oxidative damage involving singlet oxygen (Madigan *et al.*, 1997). The pigments apparently protect the colored strains against damage by ultraviolet light (David, 1973). Most mycobacterial species are whitish or cream-colored and the color is due to cytochromes and carotenoid pigments (Draper, 1983). McCready and Ratledge (1978) showed that *M. smegmatis* contains porphyrins and that the amount increases as the cells age. Porphyrins cause buff color of the cells and are present in "non-pigmented" cells. Some species have shades ranging from yellow to deep red, the color being due to carotenoid pigments (Tarnok and Tarnok, 1970). The possession of such pigments and the ability of strains to produce them either in the dark or in response to light have been used to classify some potentially pathogenic mycobacteria (Juhlin, 1967).

Mycobacteria differ from other bacteria in possessing a thick, lipid rich envelope, which gives the cells their outward characteristics, hydrophobicity, resistance to attack by macrophages in which mycobacteria are able to notify and cause disease. They have the ability to survive long periods of starvation or aridity without dehydration. Their allergic and immunogenic properties mainly originate from materials within the cell envelope, which are also instrumental in conferring drug resistance. Goodfellow and Wayne (1982) defined *Mycobacterium* as aerobic acid-alcohol fast, rod-shaped non-motile, non-sporulating organisms that contain arabinose, galactose, and mesodiaminopimelic acid in the cell wall. The guanine and cytosine (GC) base ratios in deoxyribonucleic acid are in the range of 62 mol % to 70 mol % (except *M.leprae* which has a GC base ratio of 58 mol %). Mycolic acids of high molecular weight (sixty to ninety carbons) are present, which lack components with more than two points of unsaturation in the molecule.

In mycobacteria, two basic colony types are described as ‘rough’ and smooth. While freshly isolated strains commonly grow as smooth colonies on solid media (Tsukumura, 1976) the rough mutants arise during subcultivation (Stanford and Grange, 1974). In case of *M. avium*, rough colony is more pathogenic than smooth and the smooth, opaque form is of low pathogenicity (Schaefer *et al.,* 1970).

Mycobacterial mycolic acids usually occur as a complex mixture of components that have oxygen functions such as carboxy, keto or methoxy groups, in addition to the 3-hydroxy acid system and combinations of cis or trans double bonds or cyclopropane rings; methyl branches are also encountered. The members of CMN (*Corynebacterium, Mycobacterium* and *Nocardia*) group are the only microorganisms that are able to synthesise mycolic acids. However, on the basis of the number of carbon atoms and pyrolysis esters of the mycolic acid (as well as the GC content of the DNA), discrimination among the various CMN group members is possible. Consequently, the length of the carbon backbone, the number of unsaturated links and the presence of supplementary oxygenated functions define the genus *Mycobacterium* and also the esters produced on pyrolysis (Goodfellow and Wayne, 1982).

2.5. Culture of Mycobacteria

In the early days of the bacteriology of tuberculosis, coagulated serum to which peptone, saccharose, mineral salts and glycerol was used to culture the organism. Autoclaved potato slices were sometimes used with the addition of glycerol broth. Later, egg based medium was introduced by Dorset (1903).

The early primary isolations of fish mycobacteria were achieved using Dorset and Petroff egg agar media (Aronson, 1926; Nigrelli and Vogel, 1963) and inspissated egg or oleic acid – albumin agar formulations such as Dorset egg, Lowenstein Jensen, Petragani and Middle brook 7H10 media (Dulin, 1979; Shotts and Teska 1989). The media such as Dorset egg medium, Lowenstein-Jensen (L.J) medium contain a selective agent such as crystal violet and malachite green respectively which inhibit the growth of other bacteria in addition to providing a background against which the mycobacterial colonies are more easily seen. However, their bactericidal action is very limited and various antibiotics have been added to media to make them more selective (Jenkins *et al.*, 1983).

Mycobacteria may also be occasionally isolated on general-purpose bacteriological media such as tryptone soy agar (TSA) or brain-heart infusion agar (BHIA), provided that a large inoculum is used (Sanders and Fryer, 1988). Shotts and Teska (1989) have reported the successful use of MacConkey agar for the initial isolation of rapid growing species. The most well known medium is Lowenstein-Jensen (L.J) medium. The use of sodium hydroxide to decontaminate specimens necessitates a neutralization step before the egg media could be inoculated. A buffered Lowenstein-Jensen medium, which accommodates a moderately alkaline inoculum, thus eliminates one step in the treatment of specimens (Marks and Thomas, 1958). Marks (1959) introduced highly alkaline inocula that can be inoculated directly on to acid egg medium obviating the need for neutralization and centrifugation similarly egg based media such as Dubos agar, Sauton's agar and Ogawa egg formulations have also been used successfully (Ashburner 1977; Arakawa *et al.,* 1986).

Most species of *Mycobacterium* adapt readily to grow on very simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as carbon source in the presence of mineral salts. For clinical isolates, an absolute requirement for carbon dioxide has been reported (Ratledge, 1982). Goodfellow and Wayne (1982) reported that some fastidious organisms require supplements such as hemin, mycobactins or other iron transport compounds. *M. leprae* has not been cultivated outside of living beings (Goodfellow *et al.*, 1982).

All fish mycobacteria have been cultured at ambient temperature of 20-30°C and incubation in this temperature range for 20-30 days is suitable for isolation of all species. *M. fortuitum* and *M. chelonae* are classified as rapid growers which are defined as species which provide observable growth from dilute inocula in less than 7 days incubation under optimal conditions, although *M. chelonae,* paradoxically may require several weeks incubation for primary isolation (Grange, 1981). *M. marinum* is a slow grower, which characteristically requires 7 or more days incubation for visible growth. Once isolated growth times may be reduced and the fish pathogens, in common with many other species of mycobacteria, readily adapt to growth on simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as carbon source in the presence of mineral salts (Wayne and Kubica, 1986).

Studies have shown that maximum yield of mycobacteria from environment and skin will occurs at incubation temperature of 28°C to 30°C and 33°C, respectively (Portaels, 1978). Growth rate is very slow with generation times ranging from 2 h to more than 20 h (David, 1973). Easily visible colonies are produced from dilute inoculum after two days to 8 weeks incubation at optimum temperature.

2.6. Decontamination for isolation of *Mycobacterium*

Mycobacteria may be isolated from sputum, soil, water or other sources with mixed flora only by decontaminating with agents that destroy the rapidly growing non acid-fast contaminants selectively. Krasnow and Wayne (1966) reported that time of exposure to decontaminating agents must be carefully controlled, because resistance of mycobateria is not absolute. Decontaminated specimens are inoculated to agar or inspissated egg-based medium containing malachite green, which may also be supplemented with selective anti-microbial agents.

It is very difficult to isolate *Mycobacterium* in pure culture from environmental samples because the multitudes of other microorganisms rapidly overgrow mycobacteria on the isolation media. However, taking advantage of the resistance of mycobacteria to adverse conditions, decontamination procedures and selective media have been developed to increase the efficiency of isolation procedures (Kubica and Good, 1981). Most of the decontamination methods were developed for isolation of mycobacteria from specimens of the acid-fast mycobacteria in alkaline and acidic conditions. The most commonly applied decontamination procedures involve treatment of the samples with NaOH (2-4%) for 15 to 30 minutes at room temperature or at 37°C (Jenkins *et al.*, 1983). The consequence of routinely applied decontamination procedures is that no reliable data can be obtained on the numbers of viable counts of non-decontaninated samples due to the hydrophobic characteristics of the mycobacterial cell wall, which may result in significant bacterial adhesion to surfaces (Hartmans and Bont, 1992).

2.7. Staining Method: Acid Fast Staining

An important property of certain bacteria is that of not being readily decolorized with acid-alcohol after staining with basic dye. Acid-fast bacteria include the actinomycetes, mycobacteria and some relatives, which it is believed, react in this way because of limited permeability of waxy components of the cell wall. The differential staining procedure, acid-fast staining is used to characterize a small group of organisms that stain only with great difficulty, but once stained, are difficult to destain. *Mycobacterium* is one such organism which are not decolorized with an acidic solution of alcohol. The smear is flooded with strong carbol fuchsin and heat is applied from below the slide to a bacterial smear fixed on a slide. An acidic solution of alcohol is then added to decolorize the cells; only a few groups of bacteria retain the basic fuchsin and are called acid-fast bacteria. This important acid-fastness characteristic of mycobacteria was discovered by Ehrlich in 1882. These organisms may be stained with any of a variety of basic dyes. This is the most commonly used dye for this purpose, but cannot be decolorized subsequently with dilute acids.

The acid-fast property is due to the presence of mycolic acids on the surface of the mycobacterial cells and this is found only in the genus *Mycobacterium*. It was first discovered by Robert Koch during his pioneering investigations on tuberculosis. This unique staining property permitted the identification of the organism in tuberculosis lesions. It proved to be of great taxonomic use in defining the genus *Mycobacterium.*

Acid fastness has been of immense practical importance in identifying mycobacteria rapidly, especially in pathological specimens from cases or suspected cases of mycobacterial disease. A mixture of the dye, basic fuchsin and phenol is used in this staining procedure. Acid-fast organisms in the final preparation appear red, whereas the background and non-acid-fast organisms appear blue. Mycobacteria are not readily stained by the gram method because of high surface lipid content. If the lipoidal portion of the cell is removed, it become non-acid fast but remains as gram-positive (Madigan *et al.*, 1997).

The acid-fastness lies in the ability of the dye to bind to a particular bacterial component. The substances involved in impeding the removal of the dye by acids are probably lipids, and the mycolic acid esters that form part of the bacterial cell wall seem to be the important ones (Goren *et al.*, 1978).

Aranaz *et al.* (1997) recommended using the fluorescent dyes such as auramine or acridine orange. They are superior to the conventional fuchsin stains because the mycobacteria are rendered more visible and a large area of the smear can be examined in a shorter time. Some of the scientists recommended using the modified Ziehl-Neelsen staining technique (The Fite-Faraco procedure), in which peanut oil is used with xylol at the beginning of the staining process. The addition of peanut oil limits damage to the lipid layer of the mycobacterial cell wall by the xylol.

2.8. Epidemiology

Epidemiology, the study of the prevalence and spread of the disease in a population, is poorly understood for mycobacteriosis of fish. Mycobacteriosis has been reported to affect a wide range of freshwater and marine fish species, suggesting ubiquitous distribution. (Nigrelli and Vogel, 1963; Frerichs, 1993). In fact, all fish species are considered susceptible (Bruno *et al.,* 1998). However, outbreaks predominate in tropical aquarium fish. Members of the fresh water families, anabantidae (bettas and gouramies) and characidae (danios and barbs) appear to be particularly susceptible (Astrofsky *et al.,* 2000). Intensively cultured warm water fish species including sea bass, *Dicentrarchus labrax,* striped bass, *Morone saxatilis* are also reported to be susceptible to mycobacteriosis (Hedrick *et al.,* 1987).

There have been very few reports on the occurrence of mycobacteriosis in the wild and very little is known about its prevalence and impact on wild fisheries (Heckert *et al.*, 2001). Piscine mycobacteriosis is transmitted primarily through the consumption of contaminated feed, cannibalism of infected fish or aquatic detritus (Post, 1987; Chinabut, 1990; Grady *et al.,* 1992).

Nigrelli and Vogel (1963) also suggested that the natural mode of infection may be through the ingestion of contaminated feed or by ingesting the organism directly from the water. Ross (1970) reported that guppies fed with infected chinook salmon viscera, developed lesions within two and a half months. Mycobacteriosis lesion occurring along the gut or in the skin and gills may be responsible for the release of pathogens into the water. The feeding of infected trash fish has been circumstantially shown to have disseminated the disease in cultured Pacific salmon (Ross and Brancato, 1959) and snake head fish (Chinabut *et al.,* 1990). Transmission is also thought to be possible by other means such as transovarian transmission (vertical transmission) and this has been observed in embryonic platyfish and guppies (Nigrelli and Vogel, 1963). However, Ross (1970) was unable to transmit the disease via ova and milt in steelhead trout and Parisot (1970) failed to transfer the pathogen to Pacific salmon by introducing it into the milt at spawning. Infection via the ovarian route has also been demonstrated for the viviparous Mexican platy fish (*Xiphophorus maculatus*) by Conroy (1966), but discounted as a means of spreading the disease in salmon (Ross and Johnson, 1962). Transmission from parent to offspring has also been reported, especially in the viviparous fish (Frerichs, 1993). In salmonids or other ovoviviparous fish, vertical transmission has not yet been documented (Astrofsky *et al.,* 2000).

Another route of transmission is through injuries or skin abrasions and external parasites may be involved in the cutaneous route (Post, 1987). This type of transmission of infections from lesions in skin and gills has also been considered as a mode of horizontal transmission. In this respect, potential sources of infective materials are numerous and include the soil and water in which the bacterial cells remain viable for 2 years or more (Reichenbach-Klinke, 1972; Frerichs, 1993).

Mycobacteriosis in other aquatic vertebrates may be a source of infection to fish. Frogs, snakes and turtles may become involved in the transmission cycle. Snails are also thought to be reservoir, which is especially of importance among aquarium fish (Post, 1987). Other invertebrate organisms likewise have been shown to play a role in the transmission of this agent. Grange (1985) made reference to a case of infection by *M. marinum* in aquarium fish fed with water fleas collected from a pond, which were found to harbour this bacterium. Sodjit *et al.* (1993) also demonstrated that water fleas collected from contaminated water and used as live food in the rearing of siamese fighting fish (*Betta splendens*) gave rise to characteristic granulomata of piscine mycobacteriosis within 4 months. Although there is no firm evidence to confirm that environmental stress can cause mycobacteriosis infection, Giavenni and Finazzi (1980) proposed that an unnatural environment, such as an aquarium, may actually promote the disease. Mycobacteriosis in yellow perch (*Perca flavascens*) held in an aquarium was thought to occur due to a depressed immune response in these fish (Daoust *et al.,* 1989).

2.9. Clinical characteristics and pathology of mycobacteriosis

Piscine mycobacteriosis is a systemic, chronic, progressive disease presenting various clinical features depending upon species and ecological conditions. It is presumed that fish can be infected by ingestion of feed or water contaminated feces, urine, exudates or tissues from diseased animals (Chinabut *et al.,* 1999). Mycobacteria may also infect fish through skin or gill lesions created by trauma or parasitic infection and there is even evidence that transovarian passage of the bacteria can occur (Chinabut *et al.,* 1994; Smith, 1997). After entry into the body, mycobacterial organisms spread throughout the body by the circulatory or lymphatic system (Chinabut, 1999).

If disease develops, it may include external signs such as altered pigmentation, exophthalmia, ulceration and petechiation of the skin, emaciation, cachexia, and lethargy. Internally, a common observation is the enlargement of internal organs, including the anterior and posterior kidney, spleen, liver and heart. Often, multiple white nodules can be observed grossly within these organs. These nodules are granulomas and have been reported in the brain, gills, gastrointestinal tract and skeletal muscle. If extensive granulomatous inflammation develops, the normal organ architecture can be obliterated and organ function hindered. The acute form of mycobacteriosis is often characterized by necrosis and granulomatous inflammation in internal organs. Histological examination exhibits a diffuse infiltration of macrophages and reticuloendothelial cells and the presence of mycobacterial organisms scattered throughout infected organs (Wolke and Stroud, 1978).

Formation of multiple discrete granulomas is the most distinctive feature on the lesion of chronic *M. marinum* infection in finfish. Soft granulomas are composed of centrally located caseous material in a sheath of epitheloid cells and macrophages, which are themselves surrounded by fibrous tissue (Talaat *et al.,* 1999; Wolf and Smith, 1999). Hard granulomas are formed at an earlier stage of disease and lack a defined epitheloid layer and caseous necrosis (Hedrick *et al.,* 1987). Granulomas vary in size, often ranging from 80 to 500 μm (Sakanari *et al.,* 1983) and usually contain necrotic cellular material and varying numbers of intracellular mycobacteria. Wolke and Stroud (1978) found that granulomas found in finfish differ from those found in mammals in three ways: 1) giant cells are extremely rare in finfish, 2) calcification is rarely exhibited in finfish granaulomas and 3) finfish often exhibit high numbers of acid-fast organisms within granulomas.

Wolf and Smith (1999) experimentally infected *Morone saxatilis* (striped bass) with *M. marinum* and studied the resulting granulomas. They found that multinucleated giant cells were rarely observed, mineralization was not present and acid-fast bacteria were present in significant numbers in all areas of granulomatous inflammation. These findings were supported by the studies on wild striped bass with mycobacteriosis (Hedrick *et al.,* 1987; Heckert *et al.,* 2001; Rhodes *et al.,* 2001).

2.10. Pathogenesis

M. tuberculosis is an intracellular pathogen and its pathogenicity is related to the ability to escape the killing by macrophages and the inducement of delayed type hypersensitivity (DTH). A major component of the cell wall of *M. tuberculosis* is trehalose 6,6'- dimycolate, which has been implicated as a virulence factor. The expression of DTH and cell-mediated immunity is dependent on the macrophage – cytokine – type I helper T (Th 1) lymphocyte axis. Cytokines, interleukins-12 (IL-12) and interferon – γ (IFN-γ), play a critical role in the process and IL-12-activated signal transducer and activator of transcription 4 (STAT) is required for the development of fully functional Th 1 cells (Oiso *et al.*, 2005).

An important aspect of tuberculosis infection is to understand the ability of the tubercle bacilli to invade and successfully multiply within the macrophages of the host. The macrophage, though provides the nutrients for the intracellular mycobacteria to grow, also exerts considerable environmental stress. The intracellular *M. tuberculosis* thus has adapted to the hostile environment and devised ways of withstanding or evading the stress imposed by the macrophages. *M. tuberculosis* organisms have the ability to lie dormant within this environment for prolonged periods only to be active subsequently to cause reactivation of the earlier infection in the host (Colsten *et al.*, 1999). Pathogenic mycobacteria grow inside phagosomes and phagolysomes. They are able to inhibit the fusion of bacteria-containing phagosomes and lysosomes upon macrophage infection. In the case of the tubercle bacillus, this property has been attributed to the bacterial sulpholipids that are located on the bacterial cell surfaces. Other important mycobacterial virulence factors include the lipoglycans, which are able to modulate cytokine secretion and macrophage effector function. The ability of mycobacteria to survive and modulate immune responses in the host is clearly related to the architecture of the cell envelope and the constituents contained therein (Rastogi, 1990).

The major differences between pathogenic and non-pathogenic mycobacteria are related to their ability in triggering cytokine secretion by human macrophages (Belton *et al.*, 2000). Barrow (2001) observed that unlike obligate intracellular parasites, which are completely dependent upon an intracellular environment, mycobacteria are capable of existing both intracellular and extracellularly within the infected host. In addition to intracellular survival, some mycobacteria (eg: *M. tuberculosis*) are capable of persisting in the host tissue in a state of dormant existence referred to as latency. In this state, the organism persists in an altered metabolic state, probably relying upon enzymatic pathways not used during a normal life cycle in the host.

2.11. Modern techniques for detection of *Mycobacterium* species

Definitive diagnosis of tuberculosis and non-tuberculous infections are provided only by the demonstration of the presence of mycobacteria in clinical specimens. Microscopic examination, which has been the basic method for the diagnosis of tuberculosis, requires a relatively large number of bacteria to be present in the specimens. Culture confirmation is a sensitive method for detecting mycobacteria, but it takes 3 to 8 weeks and another 2 to 4 weeks for final identification by biochemical analysis and requires expertise to interpret the results.

In the past, methods for detecting the aetiologic agent of mycobacteriosis relied on conventional bacteriology and histological examination. The stains used in histology on fixed tissue samples, were not specific *Mycobacterium* spp. In medical field, the laboratory diagnosis of *Mycobacterium* spp depends on acid-fast staining and culture of processed sputum specimens. These techniques have been continuously refined and improved also have several limitations. Microscopic examination of acid-fast smears has low sensitivity and specificity to be useful as a presumptive screening test.

Modern approaches to identify mycobacteria have been employed in recent years. It includes immunological techniques or serological tests (ELISA and Western blot), applied genetic methods (PCR and *in situ* hybridization) and physicochemical methods (Pyrolysis mass spectrometry). The BACTEC system, which detects radiolabelled carbon dioxide produced by growing organisms, can detect mycobacteria within short-term culturing. However, serodiagnosis by enzyme linked immunosorbant assay has a value similar only to that of the direct sputum smear (Daniel, 1994). Two systems, biphasic MB check and radiometric BACTEC, based on liquid media proved to be significantly better than the egg-based solid media for the isolation of mycobacteria from clinical specimens, with BACTEC being quicker to yield results (Abe, 1993).

To detect mycobacterial DNA, various methods have been reported. A simple method for the detection and identification of mycobacteria (*M. tuberculosis, M. marimum, M. scrofuloceum, M. intracellularae, M. avium, M. fortuitum,* and *M. chelonae)* uses PCR-RFLP method for the 65-kDa antigen with high sensitivity and specificity. This includes the amplification of mycobacterial genes encoding a part of 65-kDa antigen. *Hae III* restriction enzyme was found suitable for the prompt and easy discrimination among the 7 strains that was examined. Devallois *et al.* (1997) devised another PCR-RFLP method which involved the amplification of a 439 bp portion of *hsp 65*, followed by digestion with *Bst E II* and *Hae III.* The resulting data permitted the identification of 34 mycobacterial species in a single experiment.

DNA-DNA hybridization assays have been developed with DNA probes specific for rRNA's of *M. tuberculosis* complex and *M. avium-intracellularae* complex (Gonzalez and Hanna, 1987; Musiel *et al.*, 1988). These probes have greatly reduced the time required for identification, but direct detection and identification of mycobacterial species with clinical specimens are not possible. Currently PCR has been introduced as a sensitive method for detecting mycobacterial DNA directly in sputum samples.

A reliable identification may be obtained by characterizing a species-specific DNA sequence by PCR. Parra *et al.* (1991) developed a PCR technique, which amplifies a 396-bp fragment from the *M. tuberculosis* genome. Buck *et al.* (1992) reported that sonication procedure was the most effective means of treating clinical specimens containing mycobacteria to release DNA for PCR analysis.

Takewaki *et al.* (1993) developed genus specific primers that amplify the *dnaJ* gene from the broad spectrum of mycobacterial species and determined the nucleotide sequences within the *dnaJ* genes from 19 mycobacterial species on the basis of *dnaJ* gene sequences. Takewaki *et al.* (1993) also developed dot blot hybridization analysis with species-specific oligonucleotide probes for the *M. tuberculosis* complex, *M. avium*, *M. intracellularae* and *M. kansasii*, allowing rapid identification of these species following polymerase chain reaction for the *dnaJ* gene. They observed that PCR with the genus specific primer that amplifies the *dnaJ* genes and subsequent dot blot analysis with species-specific oligonueleotide probes are most useful for differential diagnosis of tuberculosis and non-tuberculous mycobacterial infections.

Various other PCR targets for *M. tuberculosis* identification include the gene encoding the *MPB 64* protein (Manjunath *et al.,* 1991), the gene encoding the 38kDa protein (Sjobring *et al.*, 1990) and the MTP 49 sequence (Portillo *et al.*, 1991); others such as DT1 and DT6 sequences allow differentiation between *M. intracellulare* and *M. avium* (Thierry *et al.*, 1993). A number of insertion sequence elements are present in numerous copies in mycobacterial genomes and are species-specific, thereby increasing the sensitivity of the assays, for example IS 6100 for the *M. tuberculosis* complex (Thierry *et al.*, 1990) and IS 1245 and IS 1311 for *M. avium* (Guerrero *et al.*, 1995).

Portillo *et al.* (1996) developed a novel multiplex PCR method with the potential to identify mycobacteria in clinical samples. The assay relies on the simultaneous amplification of three bacterial DNA genomic segments by using different sets of oligonucleotide primers. The first set of primers amplifies a 506-bp fragment from the gene for the 32-kDa antigen of *M. tuberculosis*, which is present in most of the species belonging to the genus *Mycobacterium*. The second set of primers amplifies a 984 bp fragment from the IS 6110 insertion sequence of the bacteria belonging to the *M. tuberculosis* complex. The third set of primers, derived from the *M. tuberculosis* species-specific sequence named MTP 40, amplified a 396-bp genomic fragment. Results showed that the multiplex PCR method is a rapid, sensitive and specific tool for differential identification of various mycobacterial strains in a single-step assay on the basis of the nucleotide sequence of MTP 40 species-specific protein of the tuberculosis bacillus. Definitive diagnosis of mycobacteriosis was obtained by culture on selective media followed by PCR-RFLP of the *rpo*B gene for species identification. The amplified 360-bp product of the *rpo*B gene of mycobacteria isolated from zebra fish were digested with *Msp I* restriction enzyme, which revealed unique band patterns matching those of *M. abscessus* and *M. chelonae* which are responsible for skin and soft tissues infection caused by rapidly growing mycobacteria in humans (Seok *et al.*, 2006).

The Amplicor *M. tuberculosis* test (Roche diagnostic systems, Branchborg, N.J) is a rapid, specific test that can be done directly on patient specimens, with the potential for same day laboratory results. The Amplicor PCR test uses prepared, specific reagents needed for specimen preparation, amplification and amplicon detection (Beavis *et al.*, 1995). The specimen lysis method uses a single tube system which is important for minimizing cross-contamination among specimens. Amplicor *M. tuberculosis* test amplifies a 584 bp sequence from the 16s rRNA gene. Moore and Curry (1995) showed that the Amplicor PCR can detect *M. tuberculosis* even when culture results are negative because of the treatment of patients with antimicrobiol agents or when other acid-fast organisms are isolated or when cultures are not isolated or when culture results are not available because of the overgrowth by other species of bacteria or fungi.

2.12. Treatment and Control

Treatment of mycobacteriosis in fish is difficult, partly because of availability of only two antibiotics, oxytetracycline (terramycin) and ormetoprin – sulfamethoxine are approved by the Food and Drug Administration (FDA) for use in food fish in U.S.A. (Stoffregen *et al.,* 1996). Dulin (1979) recommended the use of antimicrobials, rifampicin and isoniazid, which are commonly used against mammalian tuberculosis. Other chemotherapeutic combinations have been attempted with the use of rifampicin or cotrimoxazole plus ethambutol or rifampin plus doxycycline (Pattyn, 1984). Dujin (1981) found that water baths of tetracycline or kanamycin sulfate, respectively could help to treat the acute stages of mycobacteriosis. Lower and Poet (2001) treated external lesions related to *Mycobacterium* spp. infection with a series of enrofloxacin injections and found clinical resolution of the lesions.

Treating infected pearl gouramies (*Trichogaster leeri*) by applying penicillin to the skin of the fish and treating black widows (*Gymnocorymbus ternetzi*) with terramycin by a continuous bath have both proven successful means of treatment (Dujin, 1981). *Mycobacterium* infections in siamese fighting fish were successfully controlled by the addition of kanamycin sulphate (100 ppm) to aquarium water so as to maintain a permanent bath of the antibiotic over a 5 day period (Conroy, 1966). This antibiotic was also used successfully to control mycobacteriosis outbreaks in three-spot gouramies (*Trichogaster trichopterus* pallas) using the same treatment protocol (Santacana *et al.,* 1982). Hedrick *et al.* (1987) on the other hand found that feeding rifampin to striped bass (6mg/100g of food for 60 days) was not effective as a treatment. Colorni (1992) confirmed that mycobacteria are resistant to rifampin, whereas cycloserine reduced mortalities caused by the bacterium. Isoniazid and rifampin are recommended by Dulin (1979) as treatments for valuable exotic marine fish, against mycobacteriosis.

Antibiotics are not hundred percent effective for treating fish against mycobacterial infections. Continuous use of antibiotic drugs can also introduce drug resistant strains of the bacterium. Thus, ideal management is one of prevention meassure. Treating trash feed before use is recommended to minimize the spread of disease. The feeding of pacific salmon with pasteurized carcasses may reduce the incidence of infection (Hublou *et al.,* 1959). Dulin (1979) suggested that fish known to be infected should be burnt or buried with quicklime at a site far from water drainage. Moreover, new stocks of fish should be quarantined and screened before introduction into the hatchery or aquarium.

Reno (1998) reasoned that there are only a limited number of ways to control disease in cultured fish populations. These include reducing the spread of infectious agents within populations, chemotherapy, culling and vaccination. The potential for disease among fish depends on the contact rate among infectious agents or diseased fish and the number of susceptible fish. Under intensive aquaculture conditions, the frequency of that contact is vastly increased. Beyond over crowding and confinement, cultured fish are also subjected to other stressors such as handling, grading, fluctuating water temperatures, poor water quality and social stresses. Such factors excerbate the susceptibility of fish to disease and thus further increase morbidity and mortality in the population (Noga *et al.,* 1994).

No vaccines are available against fish-mycobacteria. But the fish are able to develop a cell-mediated immune response to mycobacterial antigens (Bartos and Sommer, 1981), which indicates a potential for the development of BCG like vaccines. Such products might find particular application in the immunization of valuable, long-lived specimens but are unlikely to be commercially justifiable for farmed food fish.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1. Sample source and type

In all 134 samples were collected which included 38 water and sediment samples from fish pond, 14 water and sediment samples from shrimp ponds of Kundapur. 10 samples of water and sediment from each of the two estuaries of Sasthan and Mulki waters, 2 sea water samples, 6 water samples from aquaria and water and sediment samples were collected from fish ponds and aquarium and 2 samples of water from swimming pool (Table 1). 500 ml of water and 100 grams of sediment were collected in new sterilized containers and brought to the laboratory for further analysis.

3.2. Isolation of *Mycobacterium* spp.

Decontamination of the samples was done before the isolation of *Mycobacterium*. Two spatula full of sediment, approximately 5 g, was taken in a sterile centrifuge tube and mixed with 30 ml of distilled water. The mixture was shaken for 30 min in a mechanical shaker to break up the clumps and suspended microorganisms. The tubes were allowed to stand for the longer particles to settle. About 10 ml of the supernatant was transferred to another centrifuge tube that contained an equal volume of the diluted hydroxide-hypochlorite solution. The tubes were shaken for 15 to 30 min and centrifuged at 2000 X g for 15 min. The supernatant was discarded and the resuspended sediment (without neutralization) was inoculated to tubes of the selective medium for *Mycobacterium* Lowenstein-Jensen (LJ) in an incubator. The tubes were incubated at room temperature (29°C ± 1°C) and at 37°C for up to 4 to 6 weeks.

Water samples were processed essentially as described above for sediment. 30 ml of water was taken and subjected to the protocol detailed above except that the sample was not placed in a mechanical shaker for 30 min.

3.2.1. Decontaminating solution (Stock solution)

Calcium hypochlorite	150 g
Sodium hydroxide	150 g
Distilled water	1000ml

The above stock solution was diluted by mixing one volume of the stock solution with six volumes of sterile distilled water before use.

3.3. Media Preparation for isolation of *Mycobacterium* sp

Fresh eggs were cleaned by scrubbing with a hand brush and soaked in soap solution for 30 minutes, following which the eggs were rinsed thoroughly in running water and soaked in 70 % ethanol for 15 minutes. The eggs were then broken gently at one end and contents collected in a sterile flask and homogenized by using sterile glass stirrer. About 18 to 24 eggs were needed to provide the required 1000 ml of homogenized whole egg to be mixed with 600ml of basal salt solution which was prepared separately.

Briefly 37.3 g of LJ (Himedia, Mumbai) medium base was suspended in 600 ml distilled water containing 12.5 ml glycerol and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 121°C (15 lb pressure) for 15 minutes and cooled to room temperature. The egg homogenate and base were mixed well and distributed in sterile screw capped tubes in volumes of 8 ml in each tube. Tubes were placed at an angle to get a slant in an inspissator. The medium coagulated in the inspissator at a temperature of 85°C for 45 minutes.

3.3.1. Basal media composition

Lowenstein Jensen Medium Base (LJ Medium Base)

Monopotassium phosphate	2.4 g/l
Magnesium sulphate	0.24 g/l
Magnesium citrate	0.60 g/l
L-Asparagine	3.60 g/l
Potato flour	30.00 g/l
Malachite green	0.40 g/l

3.4. Acid Fast Staining

The growth that came up in LJ medium were stained as follows. Slide with an air-dried and heat fixed smear was placed on a slide carrier over a trough. A piece of absorbent paper was cut to fit the slide and the paper was saturated with the carbol-fuchsin stain. The underside of the slide was carefully heated by passing a flame under the rack or by placing the slide on a hot plate until steam rises (without boiling). The preparation was kept moist with stain and steaming for 5 min, repeating the heating as needed. The film was washed with a gentle and indirect stream of tap water until no colour appeared in the effluent. The slide was washed with the decolorizing solvent followed by immediate wash with tap water. The decolorizing and washing was repeated until the stained smear appeared faintly pink. Then the smear was flooded with methylene blue counter stain for 20 to 30 sec and washed with tap water. The slides were examined using oil immersion objective. An acid-fast bacterium appeared red and non acid-fast bacterium appeared blue.

3.4.1. Acid Fast staining components

3.4.1.1. Carbol fuchsin stain

Basic fuchsin	0.3 g
Ethanol, 95 % (v/v)	10 ml
Phenol (Heat melted crystals)	5 ml
Distilled water	95 ml

asic fuchsin was dissolved in the ethanol and phenol in the water. The content were mixed allowed to stand for several days. The solution was filtered through filter paper before use.

3.4.1.2. Decolorizing solvent

Ethanol 95 % (v/v)	97 ml
Hydrochloric acid	3 ml

3.4.1.3. Counterstain

Methylene blue	0.3 g
Distilled water	100 ml

The ingredients were dissolved by shaking and stored in bottle after filtering.

3.5. Preparation of crude bacterial cell lysates for PCR

Each bacterial isolate was subcultured to fresh L.J slants. The growth on the original slant was scraped and suspended in 100 µl of DNA free distilled water taken in microcentrifuge tubes. The resulting suspension was heated at 98°C for 20 min in dry bath to lyse the cells and the tubes immediately transferred to ice for rapid release of DNA. The cell debris was settled by centrifugation at 10,000 g for 10 min and the supernatant was used for PCR. Lysates were stored at -20°C until PCR was run.

3.6. DNA extraction components

3.6.1. Reagents for DNA Extraction:

- 1 X TE buffer.
- 10 % SDS
- 20 mg/ml proteinase K (stored at –20 °C)
- 5 M NaCl
- CTAB/NaCl solution
- 24:1 chloroform, isoamyl alcohol
- 24:25:1 phenol, chloroform, isoamyl alcohol
- Isopropynol alcohol
- 70 % ethanol

3.6.2. Preparation of genomic DNA.

For genomic DNA extraction from the bacterial CTAB (Cetyl trimethylammonium bromide) method was followed. About 2 to 3 loopfuls of acid fast positive culture was suspended in 567 µl of TE (Tris-EDTA) buffer (1X). 30 µl of 10 % SDS and 3 µl of 20 mg/ml proteinase K was added. The above mixture was incubated at 45 ^{0}C for 1hr in water bath. About 100 µl of 5M NaCl was added to the tubes and mixed thoroughly. Then 80 µl of CTAB/Nacl solution was added and incubated for 10 min at 65 ^{0}C in a water bath. After incubation an equal volume of chloroform/isoamyl alcohol was added, vortexed and centrifuged at 10,000 X g for 5 min. Resulting aqueous phase was transferred to fresh tube and an equal volume of phenol/chloroform/isoamyl alcohol mixture was added and centrifuged at 10,000 X g for 5 min. The supernatant was transferred into another fresh tube and 0.6 volume of isopropyl alcohol was added and mixed gently until DNA was precipitated followed by centrifugation at 10,000 X g for 5 min. DNA pellet was washed with 1 ml of 70 % alcohol and centrifuged at 10,000 X g for 5 min. The DNA pellet was dried in a vacuum drier. Finally the extracted DNA was dissolved in 100 µl of 1X TE buffer and stored at –20°C until further use.

3.6.3. Procedure for DNA from sediment sample.

About 5 g of sediment was taken in a fresh sterile centrifuge tube containing 30 ml of distilled water. The tube was placed in a mechanical shaker for 30 min. From this about 10 ml of supernatant was taken in a separate tube and centrifuged at 10,000 X g for 10 min. The supernatant was discarded and the resulting pellet washed with 1 ml distilled water. The washed pellet was transferred to fresh microcentrifuge tube and centrifuged at 10,000 X g for 5 min. Again, supernatant was discarded and pellet resuspended in 567 µl TE buffer (1X). To this 30 µl of 10 % SDS and 3 µl of 20 mg/ml proteinase K was added and mixed properly. The tube was incubated at 45°C for 1 hr in a water bath. After incubation 100 µl of 5 M NaCl was added to the tubes and mixed thoroughly. To this 80 µl CTAB/NaCl solution was added, mixed and incubated at 65°C for 10 min, to remove polysaccharide and other contaminating molecules. An equal volume of 24:1 Chloroform: Isoamyl alcohol was mixed and centrifuged for 5 min at 10,000 X g. The resulting supernatant was transferred into a fresh tube, equal volume of 24: 1: 25 Phenol: Chloroform: Isoamyl alcohol was mixed and centrifuged at 10,000 X g for 5 min. The fresh supernatant was transferred into a separate tube and 0.6 volume of Isopropyl alcohol was mixed gently until DNA was precipitated. The precipitated DNA was centrifuged at 10,000 X g for 5 min and the supernatant discarded. Later DNA pellet was washed with 1 ml of 70 % alcohol and centrifuged at 10,000 X g for 5 min followed by drying in vacuum drier. The DNA was dissolved in 100 µl of TE buffer and stored at –20°C until use.

3.7. PCR Components

3.7.1. PCR Buffer/Assay buffer 10 X (Bangalore Genei, India)

Tris-HCl (pH 8.3)	100 mM
$MgCl_2$	20 mM
KCl	500 mM
Gelatin (w/v)	0.1 %

3.7.2. Deoxynucleotide tri phosphates (dNTPs)

dNTP mix (Bangalore Genei, India) used at 200 mM concentration of each nucleotide (dATP, dTTP, dGTP and dCTP).

3.7.3. Taq DNA polymerase

Taq DNA polymerase (0.6 units/µl) (Bangalore Genei, India) was used. The storage and dilution buffer consisted of 20 mM Tris (pH 8.0), 100 mM KCl, 1 mM DTT (Dithiothritol), 0.5 % Tween 20 (w/v) and 50 % Glycerol (v/v).

3.7.4. Oligonucleotide primers

For the detection of *Mycobacterium* sp by PCR three sets of primers were used. The first set of primers amplifies a 506 bp fragment coding gene for the 32-kDa antigen of *M. tuberculosis*, which is present in most of the species belonging to the genus *Mycobacterium*. Second set is a genus-specific primer that amplifies the *dnaJ* gene from the broad spectrum of mycobacterial species, and the Third set of primer used is a '*M. tuberculosis* complex' group specific primer.

3.7.5. 'Genus-specific' primer sequence - amplified product 506 bp

Primer	Sequence
MT1 – Forward	5' -TTCCTGACCCAGCGAGCTGCCG- 3'
MT2 – Reverse	5' -CCCCAGTACTCCCAGCTGTGC- 3'

3.7.6. '*dnaJ*' genus specific primer sequence - amplified product 236 bp

Primer	Sequence
dnaJ – Forward	5' -GGGTGACGCGGCATGGCCA-3'
dnaJ – Reverse	5'-CGGGTTTCGTCGTACTCCTT-3'

3.7.7. '*M. tuberculosis*' complex primer sequence – amplified product 383bp

Primer	Sequence
Forward TB – 1	5' -GAGATCGAGCTGGAGGATCC- 3'
Reverse TB – 2	5'-AGCTGCAGCCCAAAAGGTGT- 3'

3.8. Polymerase Chain Reaction (PCR)

3.8.1. Amplification

In a fresh, sterile PCR tube 17.1µl of sterile distilled water, 3 µl of 10 X assay buffer, 0.6 µl of 200 mM dNTP mix, 3 µl of each primer (10 pmol/ml), 0.3 µl of Taq polymerase and 3 µl template DNA solution was taken. Contents of the vial were mixed well and placed in the thermocycler (M.J. Research, USA) and programmed for required number of cycles of amplification. Each cycle consisted of denaturation, annealing and extension. The programme included an initial denaturation at 94°C for 5 min and a final delay at 72°C for 5-10 min.

3.8.2. Genus specific PCR for the Mycobacterial 32-kDa gene

Confirmation of the acid fast positive mycobacterium isolates was done by PCR technique using MT1and MT2 genus specific primers targeting the gene for 32-kDa antigen of *Mycobacterium tuberculosis*, which is present in most of the species belonging to the genus *Mycobacterium* (Portillo *et al.,* 1996). The cycling conditions used in the PCR are given below.

3.8.3. Thermocycling conditions for 32-kDa protein

Step No.	Step	Temperature (°C)	Time (min)
1.	Initial delay	95°C	5 min
2.	Denaturation	94°C	1 min
3.	Primer annealing	71°C	1.5 min
4.	Primer extension	72°C	2 min
5.	Final delay	72°C	10 min

Steps 2 to 4 × 30 cycles.

3.8.4. Genus specific PCR for the mycobacterial *dnaJ* gene

In order to detect and differentiate the major pathogenic mycobacterial species, genus-specific primers to amplify *dnaJ* gene from the broad spectrum of mycobacterial species were used as per the reports of Takewaki *et al.* (1993).

3.8.5. Thermocycling conditions for PCR of *dnaJ* gene

Step No.	Step	Temperature (°C)	Time (min)
1.	Initial delay	94°C	5 min
2.	Denaturation	94°C	30 sec
3.	Primer annealing	65°C	1 min
4.	Primer extension	72°C	2 min
5.	Final delay	72°C	5 min

Steps 2 to 4 × 38 cycles.

3.8.6. Thermocycling conditions for the group specific primer *'M. tuberculosis'* complex

Step No.	Step	Temperature (°C)	Time (min)
1.	Initial delay	95°C	5 min
2.	Denaturation	94°C	1 min
3.	Primer annealing	60°C	1-5 min
4.	Primer extension	72°C	2 min
5.	Final delay	72°C	5 min

Steps 2 to 4 × 30 cycles.

3.8.7. Detection of amplified DNA

PCR amplified products were resolved by electrophoresis in a 1.5% agarose gel to visualize the amplicons.

3.9. Electrophoresis Reagents.

3.9.1. EDTA (0.5 M) Solution

Disodium ethylene diamine tetra acetate 2 H_2O	186.1 g
Millipore water	800 ml
pH	8.0

The mixture was stirred vigorously on a magnetic stirrer and pH was adjusted 8.0 by addition of about 20 g of NaOH pellets.

3.9.2. Tris Acetate EDTA (TAE) buffer

Tris-base (Himedia, Mumbai)	48.40 g
Glacial acetic acid	11.42 g
0.5 M EDTA (pH 8.0)	20.00 ml
Millipore water	Make up to 1000 ml

A stock solution of TAE buffer (10X) was prepared by dissolving 48.4 g of Tris base, 11.42 g of glacial acetic acid and 20 ml of 0.5M EDTA (pH 8.0) in distilled water and made up the volume to 1 litre with distilled water.

3.9.3. Working solution (1X)

TAE Buffer (10X)	100 ml
Millipore water	Make up to 1000 ml

This stock solution was diluted 10 times for preparing the working solution (1X) that was used to dissolve agarose and for the tank buffer for electrophoresis.

3.9.4. Ethidium Bromide solution (2.5 mg/ml)

Ethidium Bromide	0.25 g
Millipore Water	10 ml

Ethidium bromide stock solution was prepared by adding 0.25 g of ethidium bromide (Bangalore Genei, Bangalore) to 10 ml distilled water and the mixture was stirred till complete dissolution. The solution was stored in the dark at 4°C.

3.9.5. Sample loading buffer

Bromophenol blue	0.25 g
Sucrose	40.0 g
Distilled water	100 ml

The dye was mixed with 100 ml of 40 % sucrose (w/v) and stored at 4°C.

3.9.6. Agarose Solution (1.5 %)

Agarose (Hi-media, Mumbai)	1.5 g
TAE (1X)	100 ml

1.5 g of agarose (Hi-Media, Mumbai) was added to 100 ml of 1X TAE buffer and melted in a microwave oven till the agarose dissolved completely.

3.9.7. Agarose Gel Electrophoresis

Submarine electrophoresis system (Bangalore Genei, Bangalore) was used to perform electrophoresis of the PCR products. To 1.5 % melted agarose solution prepared in TAE buffer, ethidium bromide was added after cooling to 60°C at a concentration of 0.5 µg/ml. This was poured on a horizontal gel bed, pre sealed at the sides and allowed to solidify for 30 min with the comb placed in position. After solidification, the comb and the side seals were removed from the gel bed and the solidified gel was placed in the electrophoresis tank filled with 1X TAE buffer such that the buffer was 1 mm over the gel.

About 20 µl of each PCR product mixed with 4 µl of loading buffer was loaded into the wells of submerged gel and electrophoresis was performed at a constant voltage of 120 V. About 4 µl DNA molecular weight marker (100 bp DNA ladder) mixed with loading buffer *was loaded in one of the wells and run along with the samples. When the tracking dye migrated* close to the anode, electrophoresis was stopped. The gel was observed for the bands using a DNA transilluminator and photographed using GelDoc system (Herolab, Germany) for documentation.

RESULTS

IV. RESULTS

4.1. Samples analysed for *Mycobacterium* spp. from aquatic environment.

A total of 134 different samples were analysed as shown in Table 1. From each of the thirty eight fish ponds of the College of Fisheries, Mangalore, water and sediment samples were collected. Sediment and water samples were also collected from the fourteen shrimp ponds from Kundapur near Mangalore. Sampling was also done from brackish water areas of Sasthan and Mulki estuary from where 10 samples each of sediment and water were collected. In addition samples of sea water (n = 2), aquarium water (n = 6) and swimming pool water (n = 2) were also collected for the study (Table 1).

The samples were immediately iced and brought to the laboratory for further processing.

4.2. Detection of *Mycobacterium* spp. by Zeihl- Neelsen staining of growth in LJ medium.

All the samples were processed and inoculated into LJ medium tubes in duplicate. One of the duplicate tubes of each sample was maintained at room temperature (29°C ± 1°C) while the other was incubated at 37°C. The bacterial growth in the LJ medium tubes was stained by Ziehl-Neelsen staining procedure for acid fast bacilli.

Of 38 sediment samples obtained from fish ponds of the Fisheries College, 30 yielded growth in LJ medium tubes maintained at room temperature (29°C ± 1°C). On staining, it was found to be acid fast bacteria. Only few samples showed positivity in the duplicate LJ medium tubes incubated at 37°C. From the same fish ponds, 38 water samples were also drawn. Of 38 water samples, six samples showing growth in LJ medium were positive for AFB in the tubes maintained at room temperature (29°C ± 1°C). None of the samples were positive in the duplicate tubes incubated at 37°C (Table 2).

Results in Table 2 present sediment and water samples from 14 shrimp ponds of Kundapur that were analyzed in the present study. Of fourteen sediment samples, 12 were positive for AFB by Zeihl –Neelsen staining of the growth in LJ medium tubes placed at room temperature (29°C ± 1°C) and only eight samples were positive in the duplicates incubated at 37°C. From the water samples analyzed from the same 14 shrimp ponds, 5 samples were positive by AFB staining of growth in the tubes incubated at room temperature, while none showed growth in the tubes incubated at 37°C.

10 each of sediment and water samples were obtained from the estuaries of Sasthan and Mulki. Out of the 10 sediment samples, 5 were positive for AFB by Zeihl –Neelsen staining of the growth in LJ medium tubes incubated at room temperature (29°C ± 1°C). None of the samples showed positive in the duplicates that were incubated at 37°C. Of the 10 water samples taken from the estuaries of Sasthan and Mulki, five were positive by AFB staining of growth in the duplicate tube incubated at room temperature (29°C ± 1°C) while none were positive in the duplicates that were incubated at 37°C (Table 2).

As seen from Table 2, none of the sea water, aquarium water and swimming pool water samples showed any growth in LJ Medium.

4.3. Detection of *Mycobacterium* by PCR

To confirm that the AFB positive cultures were indeed *Mycobacterium* spp, PCR was performed using both genus specific primers and *M. tuberculosis* complex group specific primers. From all the AFB positive cultures, crude lysates were prepared and subjected to PCR in a thermocycler. The crude lysates from acid fast positive tubes was expected to give a 506 bp product with MT1 and MT2 primers which are specific for the genus *Mycobacterium*. Another PCR primer pair targeting *dnaJ* region specific for *Mycobacterium* genus was expected to give a 236 bp PCR product. Combining the specific amplification of these two primer pairs, the organism could be confirmed as belonging to *Mycobacterium*.

4.4. PCR for 32kDa protein

In the case of fish pond sediment samples, incubated at room temperature, 30 samples positive for AFB staining analyzed using genus specific primers resulted in only 6 main confirmed as *Mycobacterium* spp. by PCR targeting the gene encoding 32 kDa heat shock protein present in all *Mycobacterium*.

Of 10 AFB positive culture tubes from fish pond sediment samples incubated at 37°C, 6 were positive by genus specific PCR thus confirming that the cultures were indeed *Mycobacteria.*

Of 6 fish pond water samples positive for AFB in culture, only 2 were positive by *Mycobacterium* genus specific PCR (Table 3). From 12 culture tubes, positive for acid-fast bacilli from shrimp pond sediment samples incubated at room temperature, 9 were confirmed as *Mycobacterium.* Samples incubated at 37°C yielded 8 culture tubes positive for acid fast bacilli of which 6 were confirmed as *Mycobacterium* by PCR (Table 3).

In the case of water samples obtained from shrimp ponds and incubated at room temperature (29°C ± 1°C), 5 LJ medium tubes positive for acid-fast bacilli yielded 2 confirmed *Mycobacterium* by genus specific PCR. On the other hand, the duplicate tubes of the same samples incubated at 37°C did not yield any positive acid-fast bacilli.

The estuarine sediment samples incubated at 30°C yielded 5 cultures positive for acid-fast bacilli by staining but none were confirmed to be of *Mycobacterium.* Similar was the case with estuarine water samples incubated at room temperature (29°C ± 1°C). Both the samples incubated at 37°C did not yield acid-fast bacilli in culture.

In conclusion, of 134 samples analysed, 81 showed growth in LJ medium medium tubes which was positive for acid-fast bacilli but only 31 (38.27%) could be confirmed as *Mycobacterium* by PCR targeting the 32 kDa protein (Fig. 1).

4.5. Detection of *Mycobacterium* by PCR targeting *dnaJ* gene

The positive acid-fast isolates obtained from different samples were further analyzed by genus specific PCR using a second set of PCR primers targeting a *Mycobacterium* specific *dnaJ* gene. A product of 236 bp amplicon indicates a positive reaction (Fig. 2).

Fish pond sediment samples incubated at room temperature (29°C ± 1°C) yielded 30 isolates, out of which 14 were confirmed by PCR to be *Mycobacterium.* The same samples incubated at 37°C yielded 10 isolates positive for acid fast bacilli of which 6 were confirmed to be *Mycobacterium* (Table 4).

From the fish pond water samples incubated at room temperature (29°C ± 1°C), 30 positive acid fast isolates were obtained of which 14 were confirmed to be *Mycobacterium* by genus specific *dnaJ* PCR. In the case of shrimp pond sediment samples incubated at room temperature, out of 12 positive acid-fast isolates, 9 were confirmed to be *Mycobacterium* by PCR. The same samples incubated at 37°C yielded only 8 culture positive for acid-fast bacilli of which 6 were confirmed by PCR.

Water samples obtained from shrimp ponds incubated at room temperature (29°C ± 1°C) yielded 5 positive acid-fast bacilli. Of these, only 2 were positive for *Mycobacterium* genus PCR. Similar was the case with estuarine sediment samples. From the water samples obtained from estuary none of the 5 culture tubes with growth was *Mycobacterium*.

4.6. Detection of *M. tuberculosis* complex by PCR

M. tuberculosis complex consists of *M. tuberculosis, M. bovis* and *M. africanum*. From Table 5, it can be seen that of 10 presumptively identified *Mycobacterium* from fish pond sediment by staining of the culture grown in LJ medium incubated at 37°C, six belonged to *M. tuberculosis* complex as confirmed by group specific PCR. Similarly in the case of shrimp pond sediment, six out of eight were confirmed positive and belonging to *M. tuberculosis* complex (Fig. 3). None of the other samples were positive for group specific PCR for *M. tuberculosis* complex.

4.7. Direct detection of *Mycobacterium* from environmental samples

DNA was extracted directly from the environmental samples using CTAB NaCl procedure described by Ausubel (1996). The presence of *Mycobacterium* was detected by PCR using these DNA extracts. No culture in LJ medium tubes was attempted.

Table 6 presents the results of fifteen samples of fish pond sediment and 8 samples of shrimp pond sediment analyzed in this study. From the 15 fish pond sediment samples, genus specific PCR targeting the 32 kDa heat shock protein, 6 samples gave positive bands. The second genus specific PCR targeting the *Mycobacterium* specific *dnaJ* gene detected 8 samples as positive. Of these, 4 were confirmed as belonging to the *M. tuberculosis* complex group.

From the 8 shrimp pond sediment samples, direct PCR targeting the mycobacterial specific 32 kDa protein could detect one sample (Table 6) as positive for *Mycobacterium* while the second direct PCR targeting *Mycobacterium* specific *dnaJ* gene could detect 3 samples as positive. Out of these PCR with 2 samples with group specific primers were confirmed as *M. tuberculosis* complex (Table 6).

Table 1. Source and type of samples analysed for *Mycobacterium* spp from aquatic environment

Sl.No.	Sample source	Sample type	Number of samples analyzed
1	Fish pond	sediment	38
2	Fish pond	water	38
3	Shrimp pond	sediment	14
4	Shrimp pond	water	14
5	Estuary	sediment	10
6	Estuary	water	10
7	Sea	water	2
8	Aquarium	water	6
9	Swimming pool	water	2
Total			**134**

Table 2. Detection of *Mycobacterium* spp. by Zeihl- Neelsen staining of growth in LJ Medium.

Sample source	Sample type	No. of samples analysed	No. positive by Z – N staining of growth on LJ Medium at	
			29°C ± 1°C	37°C
Fish pond	sediment	38	30	10
Fish pond	water	38	6	-
Shrimp pond	sediment	14	12	8
Shrimp pond	water	14	5	-
Estuary	sediment	10	5	-
Estuary	water	10	5	-
Sea	water	2	-	-
Aquarium	water	6	-	-
Swimming pool	water	2	-	-
Total			**63**	**18**

Table 3 . Detection of *Mycobacterium* spp. by PCR for 32kDa protein on crude cell lysates

Sample source	Sample type	PCR for 32 kDa protein with Genus specific primers No. positive /No. analysed	
		29°C ± 1°C	37°C
Fish pond	sediment	6/30	6/10
Fish pond	water	2/6	-
Shrimp pond	sediment	9/12	6/8
Shrimp pond	water	2/5	-
Estuary	sediment	0/5	-
Estuary	water	0/5	-
Total		**19**	**12**

Table 4. Detection of *Mycobacterium* spp. by PCR for *dnaJ* gene on crude cell lysates

Sample source	Sample type	PCR for *dnaJ* with Genus specific primers No. positive /No. analysed	
		29°C ± 1°C	37°C
Fish pond	sediment	14/30	6/10
Fish pond	water	2/6	-
Shrimp pond	sediment	9/12	6/8
Shrimp pond	water	2/5	-
Estuary	sediment	2/5	-
Estuary	water	0/5	-
Total		**29**	**12**

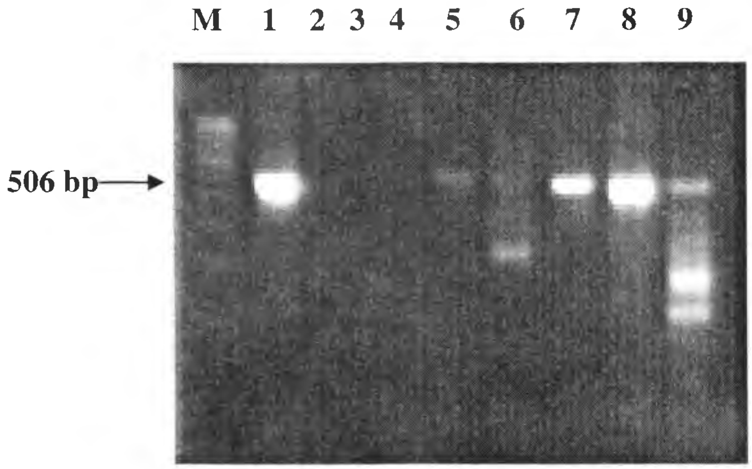
Table 5. Detection of *Mycobacterium* spp. by PCR for *M. tuberculosis* complex on crude cell lysates from 37°C incubation

Sample source	Sample type	PCR for *M. tuberculosis* complex with Group specific primers No. positive /No. analysed
Fish pond	sediment	6/10
Fish pond	water	-
Shrimp pond	sediment	6/8
Shrimp pond	water	-
Estuary	sediment	-
Estuary	water	-

Table 6. Detection of *Mycobacterium* spp. from aquatic environment by direct PCR on DNA extracts from sediment

		PCR		
		Genus specific primers		Group specific primes
Sample	No. analysed	32kDa protein	dnaJ	M. tuberculosis complex
Fish pond sediment	15	6	8	4
Shrimp pond sediment	8	1	3	2

Fig: 1. PCR amplification of 32-kDa gene using MT1 and MT2 primers



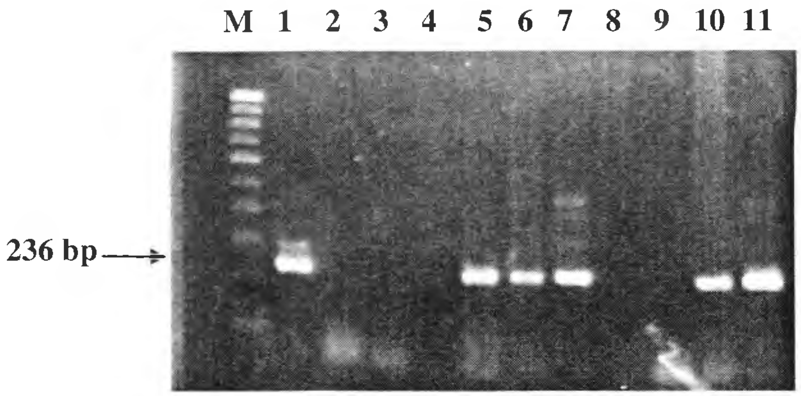
Lane M : 100 bp DNA ladder

Lane 1 : Positive control

Lane 2 : Negative control

Lane 3 to 9 : Bacterial isolates

Fig: 2. PCR amplification of *dnaJ* gene in *Mycobacterium* isolates



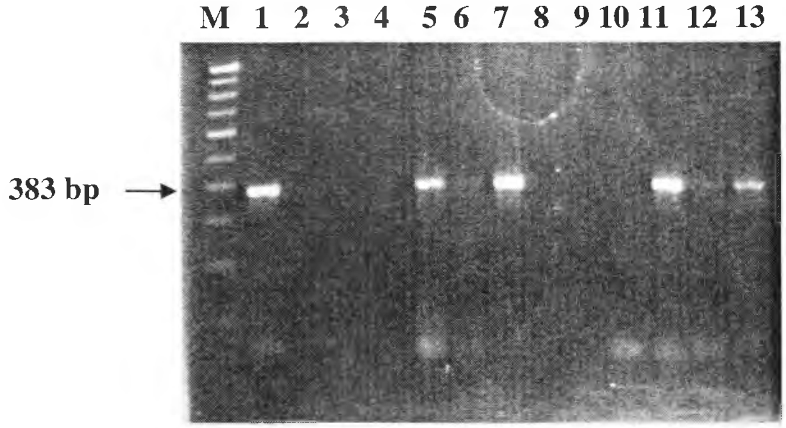
Lane M : 100 bp DNA ladder

Lane 1 : Positive control

Lane 2 : Negative control

Lane 3-11 : Bacterial isolates

Fig: 3. PCR for identification of *Mycobacterium tuberculosis* complex



Lane M	: 100 bp dna ladder
Lane 1	: Positive control
Lane 2	: Negative control
Lane 3-13	: Bacterial isolates

DISCUSSION

VI. DISCUSSION

A wide range of mycobacterial spp. have been reported in various constituents of the environment probably reflecting the ambient temperatures. In the present study, mycobacteria were isolated from sediment samples of various fishponds, shrimp ponds and estuaries. In all, 45 isolates were confirmed as *Mycobacterium* spp. by PCR out of 81 acid-fast smear positive bacteria grown on LJ medium. Twelve of the 45 PCR positive isolates of *Mycobacterium* spp. belonged to *M. tuberculosis* 'complex' which consists of *M. tuberculosis, M. bovis* and *M. africanum.*

Decontamination of samples before culture is extremely important because contaminating microflora may obscure the isolation of mycobacteria. The isolation may take a few days in rapidly growing species and several weeks in slow growing species. Incubating the medium for a prolonged period would otherwise lead to contamination. The large number of positive cultures could be attributed to the protocol adopted for decontamination.

Grange *et al.* (1990) and Pushpalatha (2004) reported that mycobacteria can be cultured from soil, especially acidic soils and areas which are marshy or prone to flooding. Grange *et al.* (1990) also suggested that particularly high numbers of mycobacteria may be found in soils rich in organic matter or heavily contaminated with animal faces. The fish ponds investigated in this study were manured with cow dung as a prerequisite step before fish culture. As domestic cattle are the natural host and reservoir for M. bovis (Lepper and Corner, 1983), it might have made its way to the sediment through cow dung. Lall (1969) has earlier reported presence of M. bovis in Indian domestic buffalo and cattle.

In this study water samples from both fish and shrimp ponds were examined and 2 isolates from each sample found positive for mycobacteria. This suggests that mycobacteria are also present in water. Many of the mycobacterial organisms naturally occur in the aquatic environment. There are few reports in which mycobacteria have been isolated from water in which diseased fish were present (Goslee *et al.,* 1976; Dailloux *et al.,* 1992). *Mycobacterium* has earlier been detected in both freshwater and the marine environment. Gruft *et al.* (1975) and Jamieson *et al.* (1976) revealed that mycobacteria can survive even in sea water.

Other 33 mycobacterial isolates which did not belong to the *M. tuberculosis* complex could be considered as atypical or non-tuberculous mycobacteria (NTM) or environmental mycobacteria. Kubica and Good, 1981 recorded that most mycobacteria, including some pathogenic species are ubiquitous environmental saprophytes. These isolates were not identified up to the species level.

This study showed that fish pond sediment and shrimp pond sediment could be a source of mycobacteria as 6 samples from each sites were found positive for genus *M. tuberculosis* complex. It is possible that these groups of bacteria may reach ponds through application of cow dung. These bacteria may be a potential source of infection for fish or fishermen.

The study also showed estuarine sediment as a source of mycobacteria, as 2 samples were found positive for genus *Mycobacterium*. It is possible that these groups of bacteria might have reached the estuary through sewage effluent and survived in the estuarine sediment. Thus estuarine sediment can become a source of infection for fish or fishermen through sewage.

Water samples of sea, aquaria and swimming pools examined in this study were negative for mycobacteria. However, the number of samples examined in this category is too small to come to any conclusions.

In the present study, attempt was also made to identify mycobacteria in soil by direct PCR, which is of great help in identifying mycobacteria from environmental sources, as mycobacteria cannot be cultured in the lab with ease (Portillo *et al.,* 1991). Amplification of DNA from soil/sediment is often inhibited by co-purified contaminants like humic acid. In this study, samples of sediment were tested for the presence of *Mycobacterium* spp. by extraction of bacterial DNA from the sediment by CTAB method. The extracted DNA was then tested with *Mycobacterium* genus specific primers and group specific primers. Initially, there was no amplification of products presumably due to the presence of inhibitors of PCR such as humic acid in the sediment. When the extracted DNA was diluted 1:10 and used for PCR, positive results were obtained. As many as 10 samples of fish pond and 3 samples of shrimp pond sediment examined by direct PCR with DNA extracted from sediment were positive for genus

Mycobacterium. Of the 13 samples positive for genus *Mycobacterium*, interestingly 2 samples from fish pond sediment and 4 samples of shrimp pond sediment were positive for *M. tuberculosis* complex. The results suggest that DNA extraction directly from sediment and water for use as template in PCR could be useful in rapid detection of mycobacteria in samples, taking into account proper methods to eliminate humic acid inhibitors of PCR.

SUMMARY

V. SUMMARY

The present study was taken up to examine the prevalence of non-tuberculous mycobacteria associated with aquatic environment and also aimed at understanding the problems due to mycobacterial species in the aquatic environment. Isolation of *Mycobacterium* spp. from water and sediment was carried out on Lowenstein – Jensen (LJ) medium followed by Ziehl-Neelsen staining of the growth that came up in the medium. This was further confirmed by polymerase chain reaction (PCR) for amplification of gene encoding a 32 kDa protein and *dnaJ* gene which was genus specific and *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis* and *M. africanum*) which was group specific.

Of 76 fishpond samples analyzed, 40 were positive for acid-fast bacilli. 24 samples were confirmed as belonging to *Mycobacterium* genus by PCR of which 6 were positive for *M. tuberculosis* complex. Other 18 mycobacterial isolates, which did not belong to the genus *Mycobacterium*, could be considered as environmental mycobacteria which are ubiquitous environmental saprophytes. Of 28 shrimp pond samples, 25 were positive by acid fast staining of growth in LJ medium tubes. Among them 17 samples were positive for *Mycobacterium* genus by PCR while only 6 were positive for *M. tuberculosis* complex group. Similarly, of 20 estuary samples, 5 yielded acid-fast bacterial growth in culture of which 2 belonged to the genus *Mycobacterium*.

Some DNA extracts of samples were also tested for the presence of *Mycobacterium* spp by PCR without attempting culture in LJ medium. Of 8 shrimp pond sediment tested, 3 were positive for genus *Mycobacterium* and 2 for *M. tuberculosis* complex. In yet another attempt on testing direct DNA extracts from 15 fish sediment samples, 10 were positive for genus *Mycobacterium* and 4 for *M. tuberculosis* complex.

The study revealed the presence of Genus *Mycobacterium* in fish pond sediment, fish pond water, shrimp pond sediment, shrimp pond water and estuarine sediment. *M. tuberculosis* complex was recorded in fish and shrimp pond sediment. PCR using cell lysates of growth in LJ medium was found suitable for specific detection of the organism. PCR on DNA extracts from samples appeared to be a suitable technique for rapid and specific detection.

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