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Sample preparation methods for isolation of *Mycobacterium* spp. from cultured fish and environmental samples

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ABSTRACT

Fish mycobacteriosis is a problem to which more than 150 species of fish are susceptible. In order to isolate non-pathogenic and fish pathogenic mycobacterial species from environmental samples, three different methods were evaluated. Shaking and membrane filtration methods were adopted to retrieve maximum acid-fast strains from fish and environmental samples. Decontamination with 4% sodium hydroxide facilitated the isolation of acid-fast bacteria in the selective media, killing all the contaminants like heterotrophs and saprophytes. Centrifugation procedure at 4000 r.p.m for 20 minutes eliminated the contaminants from the sample and allowed only acid-fast bacilli to grow in the selective media. Centrifugation was carried out twice with distilled water. Peizer TB and LJ slopes worked well for selective isolation of fish pathogenic Mycobacteria *from the centrifugated samples. It is believed that all the three sample preparation methods will be useful tool to study the fastidious fish pathogenic* Mycobacteria *from environmental samples.*

Introduction

Studies on opportunistic pathogenic bacteria were carried out extensively in Cochin backwaters and aquaculture ponds. But studies on acid-fast *Mycobacteria* from environmental samples are very few. *Mycobacteria* are ubiquitous in nature and are isolated with relative ease from fishes (Kubota *et. al.*, 1970; Land and Abernathy, 1978; Kusuda *et. al.*, 1987) from soil, stream beds, and cattle drinking troughs (Donoghue *et. al.*, 1997) and from water (Ivanainen *et. al.*, 1997; Neumann *et. al.*, 1997; Dailloux *et. al.*, 1992) has not been reported from water and sediments of aquaculture ponds.

The objective of the present study was to find out a standardised and short-cut procedure for the maximum retrieval of environmental *Mycobacteria* species from water, sediment and fish samples collected from perennial aquaculture ponds.

The normal cell morphology and bio-chemical potential of *Mycobacteria* spp. was also examined to distinguish non-pathogenic and pathogenic species especially based on lipid hydrolysis as all lipolytic forms are considered virulent. Virulence in turn is influenced by season and geographic 'niche' in which these pathogens are found. Therefore, a seasonal examination is needed on the three criteria to find out non-pathogenic and pathogenic strains of *Mycobacteria* studied, (1) pleomorphism exhibited by the stains (2) the presence of mycolic acid (3) the hydrolysis of Tween '80 ie. lipolytic strains, The above morphological and bio-chemical potential factors vary in pathogenic and non-pathogenic stains of environmental *Mycobacteria* which is suggested.

Materials and methods

In the present study, the fish, water and sediment samples were collected from perennial aquaculture ponds which were above 6 kms apart and along 9°55' - 10°10'N and 76°20'E. The perennial pond was located at Krishi Vigyan Kendra, Narakkal and other is a polyculture pond of Valappu.

Sample preparation methods for isolation of Mycobacterium

Monthly sampling was carried out from the two stations for the period from October to December 1999. The sample was brought to the CMFRI bacteriology laboratory in asceptic condition within 2 hrs for the bacteriological investigation. Fish (Tilapia - *Oreochromis niloticus*) sediment and water samples were brought to the laboratory in an ice-box (+4°C). Sediment, water as well as skin, gill, stomach, intestinal samples of fish were taken for isolation of *Mycobacteria* to know the occurrence, distribution and percentage composition of *Mycobacteria* in these samples.

In this study, the retrieval of *Mycobacterium spp.* from various samples has been standardised by incorporating methods like membrance filtration, mechanical shaking, decontamination and centrifugation procedures. The above procedures were carried out as the *Mycobacteria* are highly fastidious and won't occur easily in synthetic media. The two synthetic media recommended for isolation of *Mycobacteria* are Loewenstein - Jensen's and Peizer TB media. In the present study total heterotrophs (TPC) was also monitored using Nutrient agar to know the percentage of *Mycobacteria* in these sample. Flowchart -I shows the general procedure followed for isolation.

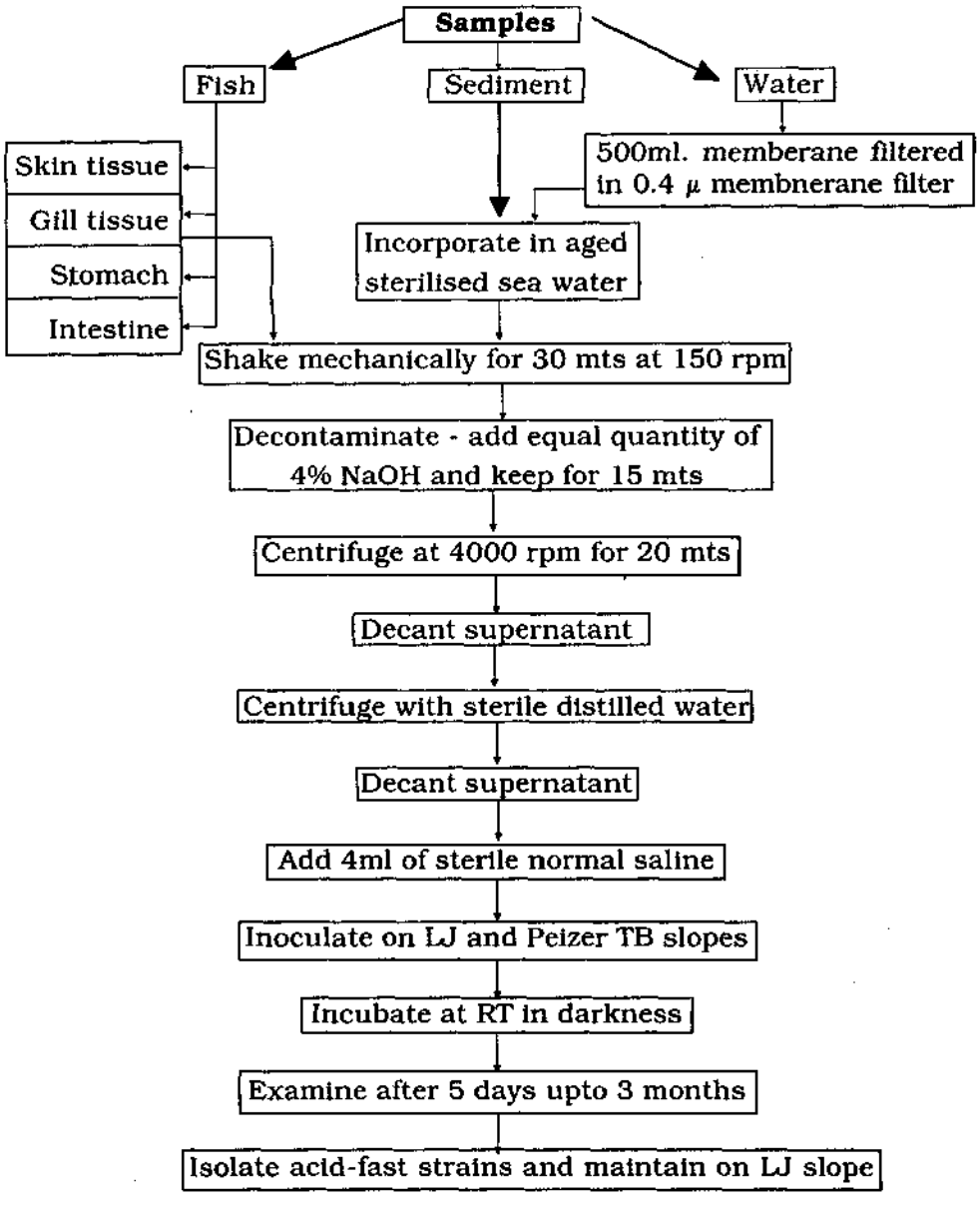
I. *Membrane filtration:* About 500 ml of water sample was membrane filtered (Whatman) using 0.4μm filters in sterile condition and TPC of heterotrophs was taken before and after the membrane filtration.

The membrane filtration technique was followed by mechanical shaking in water samples. Mechanical shaking will dismantle the adhered bacteria from the organic particles into the suspension thereby increasing the incidence of *Mycobacteria.* The decontamination procedure may vary according to the microbiologist and one of the simplest method is adopted in the present study due to its ease in its application.

II. *Mechanical shaking:* Fish and sediment samples were smashed well (1 gm each) placed in 10ml and 99 ml aged, presterilised sea water and was kept for shaking in a mechanical shaker at 150 rpm for 30 mts. The membrane filter used for the filtration of water sample was also subjected

Flow chart - I

Procedure Followed to Isolate Mycobacteria



to shaking. The data on TPC was also collected to compare the difference in total plate count before and after mechanical shaking.

III. *Decontamination procedure:* The decontamination method adopted by Marks and Thomas (1958) was followed and according to the method, 4% NaOH was poured in equal quantity of the shaken sample, mixed well and kept for 15 mts. Only the suspension was used for further procedure. The total plate count of the acid-fast bacteria are also taken before and after the decontamination procedure to understand the effect of the decontamination procedure.

IV. *Centrifugation*: The speed of cetrifugation was 3000 rpm for 20 mts. The supernatant was discarded and the centrifugation was repeated in the same speed with distilled water.

V. *Inoculation:* The supernatant of the centrifugate samples are decanted and added with 4ml of normal saline into each tube and shaken well. Duplicates of LJ and Piszer TB slopes were inoculated with 0.1 ml (for fish and sediment samples) and 0.2 ml (for water sample) of the samples.

VI. *Incubation:* Incubation was done at RT (28±2°C) and in complete darkness because some species of *Mycobacteria* were showing photoactivation capacity which was helpful for the classification by considering, the colour of the pigment produced after one hours' exposure to light. The slopes were kept in slanting portion for the first 24 hours so that the bacterial propagules may get absorbed on the slope and then kept straight, for the rest of the incubation period. In 3-5 days, colonies started to appear on slopes. The general TPC with or without NaOH procedure is also done in the same way with nutrient agar. The mycobacterial colonies on the slopes and plates are stained by Ziehl - Nelson's staining technique. Acid-fast ones were isolated for further bio-chemical and physiological studies.

Results

Membrance filtration

Mycobacteria spp. are sparsely distributed in surface water, hence sample preparation methods are suggested to retrieve maximum of them

on selective media. The membrane filtration method was adopted as an additional sample preparation method for surface pond water. The total plate count before and after membrane filtration, the TPC of aerobic hetrotrophic bacteria in two stations from October to December 99 is given in Table 1 and 2. In KVK (Table 1) the highest count obtained was during November 99 the count being 200 x 10^{-3}, and the lowest TPC encountered during October and December 1999 the count being 10 x 10^{-5}. Compared to this the TPC obtained without membrane filtration was much lower, the count ranging from 2 x 10^{-5} at the month of October to 45 x 10^{-3} at the month of November.

Total Heterotrophs (TPC) encountered in Valappu is given in Table 2. In this station, after membrane-filtration the highest and lowest values obtained were during November, the count being 36 x 10^{-3} and 10 x 10^{-4} respectively. The lowest count encountered without membrane filtration was 2 x 10^{-5}in the post-monsoon month of October 1999.

There is every possibility of obtaining more *Mycobacterium* by sample preparation methods and the incidence of *Mycobacteria* will be more in fish and environmental sample after membrance - filtration. This study will highlight the importance of these sample preparation methods in obtaining the highly fastidious *Mycobacteria* in synthetic organic media.

Table 1. Effect of membrane filtration on general TPC from water samples of KVK aquaculture pond

Months / Procedure	With filtration	Without filtration
October	10 x 10^{-5}	15 x 10^{-3}
	83 x 10^{-3}	2 x 10^{-5}
November	200 x 10^{-3}	45 x 10^{-3}
	34 x 10^{-5}	3 x 10^{-5}
December	28 x 10^{-3}	8 x 10^{-3}
	10 x 10^{-5}	4 x 10^{-5}

Table 2. Effect of membrance filtration on general TPC from water samples of Valappu aquaculture pond.

Months / Procedure	With filtration	Without filtration
October	23×10^{-3}	9×10^{-3}
	18×10^{-5}	2×10^{-5}
November	36×10^{-3}	11×10^{-3}
	10×10^{-4}	3×10^{-4}
December	21×10^{-3}	13×10^{-3}
	28×10^{-5}	6×10^{-5}

Mechanical shaking

Table 3 and Table 4 shows the count obtained before and after the mechanical shaking procedure at 150 rpm for 30 mts. The procedure was found to be effective in increasing the TPC of heterotrophic bacteria. During the sample preparation method, all the samples are subjected to this procedure and considerable difference in bacterial count was obtained in water samples.

The total heterotrophs (TPC) encountered was very high in Valappu and recorded upto 176×10^{-3} during the month of November. The TPC in the same month was only 11×10^{-3}/ml without the shaking procedure. In all the three months, the TPC was recorded high in which the lowest count recorded as 24×10^{-4}/ml in the month of November. The lowest TPC count was recorded 1×10^{-3}/ml without mechanical shaking procedure during the month of December.

Table 3. Effect of mechanical shaking on general TPC from water sample of Valappu aquaculture pond.

Months / Procedure	After shaking water sample	Before shaking water sample
October	96×10^{-3}	16×10^{-3}
	27×10^{-5}	2×10^{-5}
November	176×10^{-3}	111×10^{-3}
	24×10^{-4}	7×10^{-4}
December	39×10^{-3}	1×10^{-3}
	43×10^{-5}	12×10^{-2}

Table 4. Effect of mechanical shaking on general TPC from water samples of KVK aquaculture pond.

Months / Procedure	After shaking water sample	Before shaking water sample
October	122×10^{-3}	31×10^{-3}
	22×10^{-5}	2×10^{-5}
November	210×10^{-3}	42×10^{-3}
	40×10^{-5}	8×10^{-5}
Dcember	27×10^{-3}	3×10^{-3}
	6×10^{-5}	11×10^{-5}

Table 4, illustrates TPC of KVK, the highest count encountered was during November (210×10^{-3}/ml) and lowest during December (6×10^{-5}). In October, highest count obtained was 122×10^{-3}/ml, but the highest count before shaking was 42×10^{-3}/ml and the lowest being 2×10^{-5}/ml in October.

From this account, it is clear that there is defenite enhanced occurrence of growth in TPC and *Mycobacteria* after the shaking procedure. So it is concluded that the sample preparation methods are essential for the maximum number of *Mycobacterial* spp. retrieval from environmental samples.

Decontamination procedure

This is considered to be the most important sample preparation method as it is making the isolation of *Mycobacteria* more easy by selectively enhancing only the occurrence of *Mycobacteria* as 4% NaOH will permit only the bacteria having mycollic acid in their cell wall to grow.

Table 5 and 6 shows the TPC of *Mycobacteria* at the station of KVK before and after the decontamination procedure. All the six samples like skin, gill, stomach, intestine of tilapia and sediment and water samples were subjected to decontamination procedure.

Table 5. Retrieval of *Mycobacteria* from samples of KVK aquaculture pond before decontamination.

Months/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	-	-	5×10^{-4}	-	12.14×10^{-2} 3.7×10^{-4}	-
November	37×10^{-6} 17.2×10^{-4}	-	5×10^{-4} 2.88×10^{-6}	7.69×10^{-4} -	- -	-
December	0.85×10^{-6}	1.85×10^{-2} 1.85×10^{-4}	12.5×10^{-4}	2.6×10^{-4}	-	-

Table 6. Retrieval of *Mycobacteria* from samples of KVK aquaculture pond after decontamination.

Month/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	0.76×10^{-3}	3×10^{-4}	1.66×10^{-3} 106×10^{-5}	2×10^{-3} 1.55×10^{-5}	4×10^{-4}	-
November	-	0.86×10^{-2} 2.58×10^{-4}	14.6×10^{-3} 1.12×10^{-5}	0.90×10^{-5} 1.8×10^{-3}	4×10^{-4} 18×10^{-2}	-
December	41.4×10^{-3} 57.57×10^{-4}	0.94×10^{-4}	5.35×10^{-3}	21.6×10^{-5}	8.57×10^{-2}	-

Table 3 shows the count of TPC and *Mycobacteria* obtained in gill, stomach, intestine, sediment and water samples mycobacterial TPC in all the three months. Skin sample was showing the absence of *Mycobacteria* during November '99. The lowest *Mycobacterial* count was encountered in skin sample the count being 0.76×10^{-3}/gm during October. The skin sample harboured the highest count 37×10^{-6} and 17.2×10^{-4} during November '99. Water samples in all the three months before and after decontamination procedure is devoid of *Mycobacteria*. During December, the lowest *Mycobacterial* count observed was 0.85×10^{-6} from the skin sample.

Table 7 and 8 shows *Mycobacteria* TPC encounted at Valappu before and after decontamination procedure respectively. After decontamination, all the six samples showed mycobacterial occurrence except stomach during December. In the month of October, skin sample harboured highest TPC before decontamination procedure the count being 140.4 x

10^{-4}/gm and 130.28 x 10^{-6}. But the corresponding value after decontamination was 3.3 x 10^{-3}/gm and 9.16 x 10^{-5}/gm. In Table 7 the lowest TPC was recorded from stomach the count being 0.775 x 10^{-4}/gm. In water samples, *Mycobacteria* occurred only in the month of December '99, the count encountered as 2 x 10^{-2}/ml. The highest TPC encountered was 53.68 x 10^{-5} in skin during November and lowest was recorded during December, the count being 0.95 x 10^{-2}. In all the three months, water samples were showing mycobacterial representation, the highest count recorded during October and lowest count during December. The decontamination procedure was effective for the retrieval of *Mycobacteria* from sediments as the count obtained after decontamination was more when compared to the count obtained before decontamination, except during October'99.

Table 7. Retrieval of *Mycobacteria* from samples of Valappu aquaculture pond before decontamination.

Months/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	140.4 x 10^{-4} - 130.28 x 10^{-6}	-	.775 x 10^{-4}	2.4 x 10^{-4}	213 x 10^{-2} - 0.9 x 10^{-4}	
November	1.08 x 10^{-6}	4.16 x 10^{-4}	-	2 x 10^{-4}		
December	0.95 x 10^{-6}	6.9 x 10^{-3}	-	-	-	2 x 10^{-2}

Table 8 - Retrieval of *Mycobacteria* from samples of Valappu aquaculture pond after decontamination

Months/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	3.3 x 10^{-3} 9.16 x 10^{-5} -	10.8 x 10^{-3} 11.6 x 10-5 1 x 10^{-4}	5.5 x 10^{-3} 2 x 10^{-5} 1.7 x 10^{-5}	6.9 x 10^{-3} 3 x 10^{-6} 12.38 x 10^{-5}	2.94 x 10^{-2}	12 x 10^{-2} 16 x 10^{-4}
November	53.68 x 10^{-5}	4 x 10^{-2}	1.7 x 10^{-3}	10.6 x 10^{-3}	5 x 10^{-4}	1 x 10^{-4} 4 x 10^{-2}
December	2.06 x 10^{-3} 2.06 x 10^{-4}	0.95 x 10^{-2}	-	1.8 x 10^{-5}	32.14 x 10^{-3} 1.78 x 10^{-4}	1 x 10^{-2}

Discussion

Mycobacteriosis or fish tuberculosis is a serious infectious disease and a threat to aquaculturists. The causal organism *Mycobacterium* (the same genus of bacteria that causes tuberculosis in humans) has been isolated from different sources. Bacterial tuberculosis had been studied in yellow tails by Kubota *et. al.* (1970) and in mountain white fish by Land and Abernathy (1978). An epizootic of mycobacteriosis had been reported in yellow tails in 1987 by Kusuda *et. al.* Some mycobacteria are highly pathogenic but when isolated from environmental samples most of them are non-pathogenic. They are simply there as normal flora. Certain generalisation can be made regarding disease-causing *Mycobacteria.*

1) Most of them are aerobic.

2) All are acid-fast rod shaped or highly pleomorphic cocco-bacilli in adverse environmental conditions.

According to Dailloux *et. al.* (1992), water is the natural habitat of *Mycobacteria,* both fresh and salt water. Neumann *et. al.* (1997) has isolated *Mycobacteria* from water and they had incubated the samples on LJ slopes after enriching by filtration. It the present study, membrane-filtration procedure was adopted for making the incidence of *Mycobacterium* maximum possible. Throughout the study period, water samples were devoid of *Mycobacteria.* This may be due to the less number of mycobacterial cells which is insufficient to grow into the medium. Surface water showed high TPC in three months the count being 16 x 10^{-4}/ ml in October. Mechanial shaking was not used by any of the workers during the isolation procedure, and it is suggested that as it gives enhanced mycobacterial occurrence it may also be included in the isolation procedure.

For the decontamination procedure of the various samples, each laboratory was having their own standardised method of choice. Dalsgaard *et. al.* (1992) used the decontamination procedure by Beerwerth *et. al.* (1967) given in Procedure I. With this method, it was possible to isolate the fastidious *Mycobacteria.* After adding the decontaminant, a mixture

of 5% oxalic acid and 0.1% malachite green are added to neutralise the sample. Ivanainen *et. al.* (1997) tried two decontamination methods to isolate *Mycobacteria* from brook waters. The decontaminants used were : 0.7 mol/litre NaOH followed by 50g/litre oxalic acid and 0.9 mol/litre H Sub (2) SO Sub (4) combined with 0.5g/litre cycloheximide. The NaOH-oxalic acid method generally resulted in lower contamination and higher isolation of mycobacteria. Dalsgaard *et. al.* (1992) and Ivanainen *et. al.* (1997) used oxalic acid to neutralise the mixture. But in the present study, the neutralisation procedure was not done, the decontaminant used was 4% NaOH for 15 mts. which gave maximum retrieval of *Mycobacteria spp.*

Procedure - I

Beerwerth (1967)

Isolation procedure for *Mycobacteria*

1) Tissue treated with equal volume of 4% NaOH for 15 mts.
2) Centrifuged at 3000 rpm for 15 mts.
3) Decant supernatant.
4) Neutralise with 5% oxalic acid to which is added 0.1% malachite green for 15 mts.
5) Decant supernatant.
6) Suspend sediment in 4 ml physiological saline.
7) Inoculate 0.1 ml of this suspension on to four slants of LJ medium.

Lansdell *et. al.* (1993) isolated several *Mycobacterium* species from marine fish caught in the wild and fresh water ornamental fishes. After excising the infected tissues, the tissues were homogenized in 10 ml sterile water and an equal volume of 2-3% NaOH was added as in the present study. Each sample is mixed in a vortex mixture and allowed to remain for 15 mts at RT. Samples were centrifuged at 3000 rpm to effect 95% sedimentation rate of all bacilli present. The centrifugate was neutralised with 2NHcl. In the present observation all these methods were followed except the neutralisation procedure. Eventhough neutralisation procedure was not adopted, high counts of several *Mycobacterium* species has been recorded on Nutrient agar, LJ medium and

Peizer medium slopes.

Twelve methods for the isolation of mycobacterium were compared by Neumann *et.al.* (1997) from surface and treated waters and in each method a particular combination of decontaminants, growth medium and incubation temperature was used. The efficiency of each method was determined by calculating the positivity rate, negativity rate, contamination rate, mean number of mycobacterial colonies formed etc. It was found that 0.005% CPC was found best for treated waters. The general method adopted in the present study was found to be good for the maximum retrieval of *Mycobacteria* from water.

Isolation of *Mycobacteria* was very high using 4% NaOH as decontaminant, in all the six samples of Valappu and KVK except water sample of KVK during the study period. The absence of *Mycobacterium* in water may be due to the pH variation attained during decontamination procedure. *Mycobacteria* was absent in skin sample during November but recorded high during December. Intestine and sediment samples of KVK also showed higher values than gill and stomach.

Water samples recorded highest TPC in Valappu during the three months of study, the lowest value being $1x10^{-2}$/ml. Skin sample showed lowest count during October. Before decontamination the count encountered is $140.4x10^{-4}$ and $130.28x10^{-6}$. The corresponding count after decontamination is being $3.3x10^{-3}$ and $9.16x10^{-5}$. Sediment in October also showed high count before decontamination the count being $0.9x10^{-4}$/ gm. After decontamination, the corresponding value is only $2.94x10^{-2}$/ gm. The occurrence of highest mycobacterial count in the samples before decontamination will be quiet accidental. In the present study, both the stations were showing high mycobacterial count, after decontamination procedure.

Some of the *Mycobacteria* from environmental samples require large incubation period as they are slow-growing forms in synthetic media despite of their predominance in the environment. Diagnosis is both difficult and time consuming using conventional methods. All the sample preparation methods suggested in the present study are efficient in retrieving mycobateria from water, sediment, faeces, fishes and fish blood samples and will be useful tool in the study of both pathogenic and non-pathogenic mycobacteria from environmental samples.

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