

ISOLATION AND IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA FROM WATER AND SOIL IN CENTRAL INDIA

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Abstract

Nontuberculous mycobacteria (NTM), important organisms in the Genus *Mycobacterium* and commonly present in the environment, are known to cause disseminated disease in AIDS patients. In this study, NTM were isolated from environment (soil and water) of the AIDS patients with disseminated NTM disease to know the prevalence of environmental NTM species and their correlation with clinical isolates from patients of the same area. Paraffin baiting technique was used to isolate NTM from environmental samples. Once isolated, subcultures were made on Lowenstein Jensen and Middlebrook 7H10 media and the species were identified using phenotypic and genotypic techniques. A total of 26 NTM isolates belonging to seven different species could be identified. *Mycobacterium avium* was the only species isolated from both clinical and environmental samples of the same patient; but the isolates did not match using PCR for IS 1311 and IS 1245 spacer sequences.

Key words: Central India, environmental mycobacteria, nontuberculous mycobacteria

Introduction

Nontuberculous mycobacteria (NTM), also known as environmental mycobacteria are found mainly as saprophytes in the environment. They may infect human beings either by ingestion or inhalation and have a two-fold effect. Firstly, in general population they may affect the efficacy of BCG^[1] and depending on the nature of the NTM, their exposure can enhance, mask, or interfere with the efficacy of subsequent BCG vaccination.^[2] Secondly, NTM can cause mycobacteriosis in immunocompromised patients especially those with AIDS and present as disseminated disease.^[3] Isolation of NTM from the environment reveals the epidemiological distribution in a particular region, which is useful in interpreting the efficacy of BCG or to know the species that might lead to disease in AIDS patients in that area. So far, only two studies, one each from South^[4] and North India^[5] have documented isolation of NTM in the environment. As many contaminating bacteria are also present in the environment, a number of decontamination techniques have been utilized in the past to isolate NTM on Lowenstein Jensen medium.^[5,6] A selective medium in this regard would have been very useful.

In our laboratory, we have standardized a system that is based on “paraffin baiting” and acts like a selective medium for NTM.^[7] Paraffin baiting is based upon the principle that

paraffin utilizing organisms or ‘paraffinophilic organisms’ are able to utilize paraffin wax as a sole source of carbon when placed in a basal salt medium (Czapek Broth), which is devoid of any other carbon source. In this system, a paraffin coated slide dipped in the broth serves as the sole source of carbon. Paraffin baiting technique permits growth of very limited organisms like *Nocardia*, NTM and *Rhodococcus* among the acid fast group. It may get contaminated with *Pseudomonas* and *Candida* species for which it is made inhibitory by the addition of BACTEC PANTA Plus.^[8] This technique was evaluated in our laboratory for NTM isolation from clinical samples in two different studies.^[9,10]

The present study was performed to isolate NTM, using paraffin baiting technique, from the environmental samples of AIDS patients with mycobacteriosis in central India.

Materials and Methods

Environmental samples viz. soil, drinking water, and water from other sources were collected for the study. These samples were collected from the household and work area of five AIDS patients in whom NTM were isolated from clinical samples like blood and stool in another approved study.^[11] It involved five villages as given in Table 1.

Processing of environmental samples:

Wet soil samples of approximately 5g were collected from a depth of 3cm. Water samples, 50ml each, were collected from drinking water sources as well as ditches and ponds. Soil (total amount) was suspended in 20 ml of double-distilled autoclaved water (D/W) in polycarbonate centrifuge tubes. After shaking manually for 60 sec, the suspension was centrifuged at 600×g for 5 min at room

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Table 1: Village wise isolation of NTM from clinical and environmental samples

Village. No.	Clinical NTM isolate	Environmental NTM isolates	
		Soil	Water
1	<i>M. avium</i> =1	<i>M. fortuitum</i> =4 <i>M. chelonae</i> =1 <i>Nocardia</i> sp.=2	<i>M. fortuitum</i> =1
2	<i>M. avium</i> =2; <i>M. wolinskyi</i> =1	<i>M. avium</i> =1 <i>M. chelonae</i> =1 <i>M. flavescens</i> =1 <i>M. phlei</i> =1	<i>M. fortuitum</i> =1
3	<i>M. simiae</i> =2	<i>M. avium</i> =1 <i>M. fortuitum</i> =2 <i>M. abscessus</i> =1 <i>Nocardia</i> sp.=1	<i>M. abscessus</i> =1
4	<i>M. vaccae</i> =1	<i>M. fortuitum</i> =1 <i>M. abscessus</i> =1 <i>Rhodococcus</i> sp.=1	<i>M. fortuitum</i> =1 <i>M. flavescens</i> =1
5	<i>M. simiae</i> =1	<i>M. avium</i> =2 <i>M. fortuitum</i> =1 <i>M. chelonae</i> =1 <i>M. thermoresistibile</i> =1	One isolate from drinking water was lost on subculture

Villages: 1. Sawangi District Wardha, 2. Karanji Bhoge District Wardha, 3. Nandori District Wardha, 4. Wakhed District Wardha and 5. Wani District Chandrapur

temperature (RT 23°C) to pellet the soil particles. The turbid supernatant (10 ml) was transferred into other sterile centrifuge tubes and centrifuged at 8,000×g for 15 min at RT.^[5]

Water samples (total volume) were centrifuged at 8,000×g for 15 min at RT and the supernatant was discarded.^[5]

Pellets from the soil and water samples were re-suspended separately in 20 ml of 2% NaOH^[12] and incubated at room temperature for 30 min. After incubation the suspensions were centrifuged at 8,000×g for 15 min at RT, and the supernatants were again decanted. The pellets were washed twice with 20 ml of D/W and finally re-suspended in 2 ml of D/W. One ml of the suspensions was then added to tubes containing paraffin coated slides (Designer Diagnostics™, Designer Diagnostics, 1930 Village Center Circle 3-947, Las Vegas, NV 89134) in about 4ml Czapek broth (NaNO₃ 3.0g, K₂HPO₄ 1.0g, MgSO₄.H₂O 0.5g, KCl 0.5g, FeSO₄ 0.01g, Distilled Water 1000ml, pH 7.5; all reagents purchased from HiMedia Mumbai, India) containing 0.ml of BACTEC PANTA Plus (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) (Becton Dickinson India Pvt. Ltd Mumbai, India). This system was labelled as Paraffin Slide Culture.

Once the growth was observed on the paraffin slides, it was stained with Ziehl Neelsen technique and subcultured on Lowenstein Jensen and Middlebrook 7H10 agar (BBL Difco, distributed by Becton Dickinson India Pvt. Ltd Mumbai, India) media and processed further by staining

and identification using phenotypic and genotypic methods. Following phenotypic tests were performed in the laboratory for identification - temperature preference (°C), growth at 42°C, growth at 52°C, growth rate, pigment production in dark and on exposure to light, growth on PNB, growth on MacConkey, growth in the presence of 5% NaCl, niacin, semi-quantitative catalase (mm), heat stable catalase, nitrate reduction, tellurite reduction, tween 80 hydrolysis, aryl sulphatase three days, aryl sulphatase 14 days, urea hydrolysis, utilization of citrate, mannitol, and inositol, iron uptake and pyrazinamidase.^[13] Genotypic tests were PCR restriction analysis of hsp65 genes using *HeaIII* and *BstE2* restriction enzymes.^[14] *M. avium* isolates were typed using PCR for IS 1311 and IS 1245 spacer sequences.^[15]

Results

A total of 60 environmental samples, 20 each of soil, drinking water and water from other sources, were collected from 5 villages of patients diagnosed with disseminated NTM disease. The clinical isolates from the patients were *M. avium*, *M. simiae*, *M. wolinskyi* and *M. vaccae*. [Table 1]

A total of 26 environmental NTM isolates were recovered during the study, including 20 from soil (*M. avium* 4, *M. fortuitum* 8, *M. chelonae* 3, *M. abscessus* 2, *M. flavescens* 1, *M. phlei* 1 and *M. thermoresistibile* 1); 5 from water sources other than drinking (*M. fortuitum* 3, *M. abscessus* 1 and *M. flavescens* 1) and a single unidentified isolate from drinking water. [Table 1] Thus a total of seven species of NTM were isolated including both slow and rapidly growing mycobacteria.

Multiple isolates were also recovered from soil samples – one sample had three isolates and three had two isolates each [Table 2].

Isolation rate was 75% from soil samples and 25% from water samples. [Table 2].

In the village number 2, *M. avium* was isolated from both clinical (two isolates, one each from blood and stool sample of the same patient) and environmental (one isolate from soil) sample. In addition, four more isolates of *M. avium* were recovered during the study. Genotyping using PCR for IS 1311 and IS 1245 spacer sequences for these seven strains showed that there was no correlation among them except for two clinical isolates of the same patient mentioned above.

Along with NTM, *Nocardia* was recovered in three soil samples and *Rhodococcus* in one. [Table 1] Contamination with *Pseudomonas* was observed in three water samples.

Discussion

Two approaches in the past have been used in investigating the environment for nontuberculous mycobacteria. The first has entailed straight forward analysis of the environment using various culture techniques i.e. pure ecological mycobacterial studies. Such studies have shown that mycobacteria are ubiquitous in nature, a fact that has been known since around the turn of the century. One such study was also performed in North India^[5] in which, *M. avium*, *M. kansasii*, *M. terrae*, *M. marinum*, *M. fortuitum*, and *M. chelonae* were isolated from water and *M. avium*, *M. terrae*, *M. fortuitum* and *M. chelonae* were isolated from soil samples.

The second method of approach is to work backwards from the human isolates and to look for evidence of existence of the same mycobacteria in the environment^[4] and this was done in the present study also. Such studies may throw light on the epidemiology of the disease and

could help to relate the mode of infection. We had isolated *M. avium*, *M. simiae*, *M. vaccae* and *M. wolinskyi* from clinical samples of AIDS patients^[11] and wanted to see if the same NTM species were present in their environment. Our environmental isolates included *M. avium* and a number of rapidly growing NTM species as mentioned in Table 1. However, in none of the cases same species was recovered from the patients and their environment except in one case where *M. avium* was isolated both from patient and his environment, but genotypically the strains were found to be different. In another study from BCG trial area in south India, the isolation profiles of environmental mycobacteria (soil, water and dust samples) and those isolated from clinical samples (sputum) of chest symptomatics were compared. Isolates belonging to the *M. avium-intracellulare-scrofulaceum* complex were found to be predominant in water, dust and sputum samples, while *M. fortuitum* complex organisms were predominant in soil samples.^[4] The isolates were not typed and further correlation was not studied.

The prevalence of various NTM species in the environment of India in three different regions might suggest that these bacteria should have been the main isolates from AIDS patients, however, the situation is not so and only very few studies have demonstrated isolation of NTM in such patients.^[9,10,16] This might be due to the fact that possibly our AIDS patients die of other causes before they reach a stage at which nontuberculous mycobacterial disease develops, as has also been suggested for African AIDS patients.^[12]

The clinical and environmental isolates of *M. avium* were correlated using molecular technique i.e. PCR for IS 1311 and IS 1245 spacer sequences and no correlation was found as mentioned in the results. Only two isolates from blood and stool of one patient were found to be the same. This could have been because of the fact that the environmental samples were collected after we had recorded growth of NTM in the clinical samples. Another study in the past had also tried to investigate this correlation.^[17] In this study from San Francisco, the authors looked comparatively at environmental sources (food, water and soil) in homes of persons with dual HIV and NTM infection. With use of serotype and multilocus enzyme electrophoresis analysis, some of the soil isolates were found to be similar to isolates recovered from study patients and it was concluded that soil, rather than water was the predominant environmental reservoir.^[17]

To conclude, *M. avium* and various other NTM were isolated from environmental samples. However, the correlation between environmental and clinical isolates could not be detected.

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Table 2: Isolation rate of NTM from environmental samples

Environmental source	NTM isolates	Isolation rate
Drinking water (n=20)	1@	5%
Other water source (n=20)	5	25%
Soil (n=20)	Single isolates – 11 samples Two isolates – 3 samples* Three isolates – 1 samples**	75%

@Isolate could not be revived for further processing.

M. fortuitum* & *M. chelonae*; *M. flavescens* & *M. chelonae*; and *M. fortuitum* & *M. abscessus*; *M. avium*, *M. fortuitum* and *M. thermoresistibile*

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