Nontubercular Mycobacteria in drinking water of some educational institutes in Jabalpur (M.P.), India

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Abstract Sixteen isolates of Nontubercular Mycobacteria species were isolated from drinking water supply of some educational institutes in Jabalpur during July 2006 and were identified by biochemical test, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis and PRA (PCR restriction enzyme analysis) of *rpoB* gene. Out of 21 water samples total 16 isolates of nontuberculous mycobacteria were identified, as *M. terrae* (6), *M. szulgai* (4), *M. goodii* (3), and one each as *M. malmoense*, *M. kansasii*, and *M. gastri*.

Key words Mycobacteria · Drinking water · HPLC · PRA

In recent years, Nontubercular Mycobacteria (NTM) has emerged as a major cause of opportunistic infection in immunocompromised persons especially in AIDS patient¹. NTM has known to be associated with human², animals³, soils⁴ and water^{5,6}. In 1997, Hunter⁷ reported that about eight species of mycobacteria has been associated with water borne transmission of human disease. Today the list continues to grow with the addition of potential pathogenic

mycobacteria species whose transmission is associated with water.

The NTMs have recently attracted attention for two reasons. Firstly, wherever tuberculosis has declined within the population at large they now account for greater proportion of causes of *Mycobacteria* diseases. Secondly, like tuberculosis they have emerged as common secondary infection among patients with AIDS⁸.

The NTMs produce both pulmonary and extrapulmonary infections. The isolation rate of NTM ranged from 8.3–28% from Northern India⁹. There are several other Indian reports. Katoch et.al.¹⁰ reported a case of lepromatous leprosy that was found to have chest infection due to *M. fortuitum*. Varied infection of Mycobacteria has been reported from Amritsar¹¹, Bangalore¹² and Chandigarh¹³.

The CDC estimated that 1.8 out of 100000 individual are infected with non-AIDS related mycobacteria disease each year in the USA (In the USEPA's (U. S. Environmental Protection Agency) mycobacteria: Drinking water fact sheet). In the present study was undertaken to examine the presence of Nontubercular mycobacteria in drinking water supply of some school and colleges of Jabalpur region.

Materials and Methods

Sample Collection

Water samples were collected in sterilized bottles from drinking water supply of selected schools and colleges of Jabalpur city and brought to the laboratory under ice cold condition, stored in refrigerator and processed within 24 hours. The selected schools and colleges

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were:- Kendriya Vidyalaya of Gun Carriage Factory No. 1, Kendriya vidyalaya of Gun Carriage Factory No. 2, Kendriya vidyalaya C.O.D, Gun Carriage Factory School, Government Science College, Government Mahakoshal Arts and Commerce College, Govindram Seksaria College, Nuvyug Arts and Commerce College, Leonard High School, St. Thomos High School, St. Aloysius Senior Secondary School, M.G.M Higher Secondary School, K.B.M Higher Secondary School, Shri Gurunanak Mission School, N.E.S Science College, Sanatam Dharm Public Higher Secondary School, Hitkarni Primary School, St. Paul Higher Secondary School, Shishu Shiksha Mandir Higher Secondary School, Guru Gobind Singh Khalsa School and Holy Public School.

Isolation of Mycobacteria

Mycobacteria were isolated from the water samples after decontamination of water samples by using 3% sodium lauryl sulfate (SDS) in combination with 1% sodium hydroxide (NaOH)¹⁴. The sediment obtained after decontamination of water samples were streaked on Lowenstein-Jensen (LJ) slopes in duplicates and incubated at 37°C in dark and examined on alternate days for the first 10 days and then once a week upto 3 months. Colony morphology was

recorded and acid fast staining was performed. Morphological and cultural characteristics along with growth rate and biochemical tests were performed on acid fast bacilli (AFB) strains following the method of Wayne and Kubica¹⁵.

Identification of Mycobacteria

For identification of mycobacterial species *mycolic acid* analysis of cell-wall was done using thin layer chromatography (TLC)¹⁶ and high performance liquid chromatography (HPLC)¹⁷ For TLC mycolic acid were extracted and analyzed following the method of Hamid et.al¹⁶ and the spot patterns obtained were matched with standard strain. Similarly for HPLC mycolic acids were extracted and analyzed following the method of Butler et al.¹⁷, and the chromatogram results were matched with standard strains obtained from National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, (U.P) India.

Further confirmation of TLC and HPLC result were done by PRA (PCR restriction enzyme analysis) of *rpoB* gene (360bp). Primer set consisting of 5'CAAGGAGAAGCGC-TACGA-3' (RPO5') and 5'-GGATGTTGATCAGGGTCTGC-3' (RPO3') resulted in 360-bp PCR products. PCR was carried out in a final volume of 50 ml consisting of 10 ml of DNA supernatant containing approximately 10 ng

Table 1 Biochemical test for Identification of nontuberculous Mycobacterium species isolated from drinking water supply of some educational institutes in Jabalpur.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
BGCC# 455	+	<7	S	+	-	+	+	+	-	+	-	+	Smooth & Yellow	-	<i>M. szulgai</i>
BGCC# 456	+	<7	S	+	-	+	+	+	-	+	-	+	Smooth & Yellow	-	<i>M. szulgai</i>
BGCC# 457	+	<7	S	+	-	+	+	+	-	+	-	+	Smooth & Yellow	-	<i>M. szulgai</i>
BGCC #458	+	<7	S	+	-	-	+	+	-	+	-	+	Smooth & Yellow	-	<i>M. gordonae</i>
BGCC# 459	+	<7	S	+	+	+	+	-	-	+	-	+	Rough & White	-	<i>M. malmoense</i>
BGCC# 460	+	<7	S	+	-	-	+	+	-	+	-	+	Rough & White	-	<i>M. gordonae</i>
BGCC# 461	+	<7	S	+	-	+	+	+	-	+	+	+	Smooth & Yellow	-	<i>M. kansasii</i>
BGCC# 462	+	<7	P	+	-	+	+	+	-	+	-	+	Smooth & Yellow	-	<i>M. szulgai</i>
BGCC# 463	+	<7	S	+	-	+	+	-	-	+	+	+	Smooth & Yellow	-	<i>M. gastri</i>
BGCC# 464	+	>7	S	+	+	-	-	-	+	+	+	+	Smooth & Yellow	-	<i>M. terrae</i>
BGCC# 465	+	>7	S	+	+	-	+	+	-	+	+	+	Smooth & Yellow	-	<i>M. terrae</i>
BGCC# 466	+	>7	S	+	+	-	+	-	+	+	-	+	Smooth & Yellow	-	<i>M. terrae</i>
BGCC# 467	+	>7	S	+	+	-	+	+	-	+	+	+	Smooth & Yellow	-	<i>M. terrae</i>
BGCC #468	+	>7	S	+	+	-	+	-	+	+	-	+	Orange and Smooth	-	<i>M. terrae</i>
BGCC# 469	+	>7	S	+	+	-	+	+	-	+	+	+	Orange and Smooth	-	<i>M. terrae</i>
BGCC #470	+	<7	S	+	-	-	+	+	-	+	-	+	Smooth & Yellow	-	<i>M. gordonae</i>

1-Code 2-AFB (acid fast bacilli) 3-Growth rate (<7 Less than-7day, >7 more than-7day) 4- Chromogenic property (S- Scrotochnomgen, P- photochromogen) 5- Tween Hydrolyses 6-Tellurite Reduction 7-Urease Test 8- Heat Stable Catalase 9-Nitrate Reduction 10-Iron uptake 11-NaCl (Sodium chloride) tolerance 12-Growth on MacConkey 13-Arylsulfatase 14-Colour and texture 15-Niacin Test 16-Identification, BGCC-(Bacterial Germplasm Culture Collection).

Fig. 1 (A, B, C, D) Mycolic acid profile of nontuberculous Mycobacterium (isolated from drinking water of some educational institute of Jabalpur) by TLC.

of genomic DNA, 10 pmol of each primer, 2 mM $MgCl_2$, 200 mM deoxynucleoside triphosphates, and 1 U of Taq DNA polymerase. DNA samples were first denatured completely by incubation at 94°C for 5 min before the amplification cycle; then amplified by subjecting it to 35 cycles of (i) denaturation at 94°C for 1 min, (ii) primer annealing at 58°C for 1 min, and (iii) elongation at 72°C for 1 min. After the last amplification cycle, the samples were incubated further at 72°C for 7 min for complete elongation of the final PCR products. After successful amplification of

the 360-bp PCR product was confirmed, and were subjected to restriction enzyme digestion using *Msp*-I and *Hae*-III enzymes by following the method of Hyeyoung lee et al.¹⁸.

Results and Discussion

Water samples were collected from drinking water supply of 21 different schools and colleges of Jabapur city. Except few institutes (Kendriavidyalaya of GCF No.2, KBM Higher

secondry school, Shri Gurunanak Mission school, NES Science college, Guru Gobind singh Khalsa School) the presence of mycobacteria was noticed from all other institutes including in present studies. Total 16 isolates were presumptively identified as member of genus *Mycobacterium* on the basis of colony morphology on Lowenstein-Jensen egg media slopes and Ziehl-Neelsen staining.

Acid fast bacilli (AFB) isolates were identified upto species level on the basis of Growth rate, Morphological characteristic, Colony characteristic and Biochemical characteristics, with the help of Bergey's manual of systematic bacteriology and confirmed by Probabilistic Identification of Bacteria (PIB) computer kit (Table 1). Out of 16 isolates of nontuberculous mycobacteria 6 were identified as *M. terrae*, 4 as *M. szulgai*, 3 as *M. gordonae*, and one each as *M. malmoense*, *M. kansasii*, and *M. gastri*.

Mycolic acid profile was investigated using TLC and HPLC and results are represented in Fig.1 and Table 2. The number of spots and there R_f values were calculated and matched with reference strain (Fig. 1, Table 2). The HPLC results of mycobacterial species isolated from

drinking water sources have been presented in Fig.2 A-F. The chromatogram results (based on cluster pattern, number of peaks and retention time of mycolic acid) were matched with the reference strain, and it was found to be: *M. gordonae* (Fig. 2A), *M. kansasii* (Fig.2B), *M. malmonse* (Fig. 2C), *M. szulgai* (Fig.2D), *M. terrae* (Fig. 2E), and *M. gastri* (Fig.2F). These were further confirmed by PCR-restriction fragment length polymorphism analysis (PRA) of *rpoB* gene. (Fig.3, Table 3).

It was observed that *M. terrae* was the most prevalent mycobacterial species in water samples of St.Aloysius Senior Secondary School, M.G.M Higher Secondary School, Sanatam Dharm Higher Secondary School, Hitkarni Primary School, St.Paul Higher Secondary School and Shishu Shiksha Mandir School. *M. terre* is a saprophyte and had also been previously isolated from public potable water supply^{19,20}. *M. terrae* had also been reported from tap water³, sputum and gastric lavage specimens from humans²¹ and soil²².

The other predominating species was *M. szulgai*, which was found in water samples of Kendriya Vidyalaya of

Table 2 R_f values of mycolate on TLC & HPLC cluster pattern of mycolic acid chromatograms from nontuberculous mycobacterium isolated from drinking water supply of some educational institutes.

Lane	Isolate No.	Isolates	No. of spots on TLC plates	R_f values				HPLC cluster	No. of Peaks
				Spots 1	Spots 2	Spots 3	Spots 4		
1	Ref. strain	<i>M. gordonae</i>	4	92.8	74	65.2	38.1	Single	6
2	Ref. strain	<i>M. malmonse</i>	3	83.2	70.1	62.1	-	Double	11
3	Ref. strain	<i>M. terrae</i>	4	91.1	69	67.2	42.1	Double	9
4	BGCC#464	<i>M. terrae</i>	4	91	69	67.1	42.1	Double	9
6	BGCC#465	<i>M. terrae</i>	4	91.1	69	67.1	42	Double	9
7	BGCC#466	<i>M. terrae</i>	4	91.1	69.1	67.1	42.1	Double	9
8	BGCC#459	<i>M. malmonse</i>	3	83.1	70.1	62	-	Double	11
9	BGCC#467	<i>M. terrae</i>	4	91.1	69.1	67	42	Double	9
10	BGCC#468	<i>M. terrae</i>	4	91.1	69	67.1	42	Double	9
12	BGCC#469	<i>M. terrae</i>	4	91	69.1	67	42.1	Double	9
13	Ref. strain	<i>M. szulgai</i>	4	92.6	70	65	32.1	Single	8
14	BGCC#455	<i>M. szulgai</i>	4	92.5	70	65.1	32	Single	8
15	Ref. strain	<i>M. kansasii</i>	2	92.4	84.1	-	-	Single	7
16	BGCC#456	<i>M. szulgai</i>	4	92.5	70	65	32	Single	8
17	BGCC#457	<i>M. szulgai</i>	4	92.5	70	65.1	32	Single	8
18	BGCC#462	<i>M. szulgai</i>	4	92	70	65	32.1	Single	8
19	BGCC#461	<i>M. kansasii</i>	2	92.4	84.1	-	-	Single	7
20	BGCC#458	<i>M. gordonae</i>	4	92.6	74	65	38.1	Single	6
21	BGCC#460	<i>M. gordonae</i>	4	92.8	74	65	38	Single	6
22	BGCC#470	<i>M. gordonae</i>	4	92.5	74.1	65.2	38.1	Single	6
24	Ref. strain	<i>M. gastri</i>	1	92.8	-	-	-	Single	7
25	BGCC#463	<i>M. gastri</i>	1	92.7	-	-	-	Single	7

Fig. 2 (A) High Performance liquid chromatogram of *M. gordonae* (BGCC #458) from drinking water. (B) High Performance liquid chromatogram of *M. kansasii* (BGCC #461) from drinking water. (C) High Performance liquid chromatogram of *M. malmoensee* (BGCC #459) from drinking water. (D) High Performance liquid chromatogram of *M. szulgai* (BGCC #455) from drinking water. (E) High Performance liquid chromatogram of *M. terrae* (BGCC #469) from drinking water. (F) High Performance liquid chromatogram of *M. gastri* (BGCC #463) from drinking water.

G.C.F No.1, Kendriya Vidyalaya C.O.D, Leonard High School, and Gun Carriage Factory School. *M. szulgai* was isolated from potable water sample by Chang *et al.*²³. This organism is associated with pulmonary disease²⁴, cervical adenitis and olecranon bursitis and keratitis²⁵ in man.

M. gordonae was isolated from water samples of Gun Carriage Factory School, Govindram Seksaria College, and Holy Public School. *M. gordonae* is known as “the water bacillus”, “the tap-water bacillus”, and “the tap-water scotochromogen”. Skin infection caused by *M. gordonae* occur

Fig. 3 Results of PRA of *rpoB* gene of mycobacterial strains. Primer pair RPO5 and RPO3 was used. Amplified DNA was digested with *Hae*III restriction enzyme. MW (Mol. Wt. marker), lane 1 (BGCC#458), lane 2 (BGCC#460), lane 3 (BGCC#470), lane 4 (BGCC#455), lane 5 (BGCC#456), lane 6 (BGCC#457), lane 7 (BGCC #462), lane 8 (BGCC#461), lane 9 (BGCC#464), lane 10 (BGCC#465), lane 11 (BGCC#466), lane 12 (BGCC#467), lane 13 (BGCC#468), lane 14 (BGCC#269), lane 15 (BGCC#459), lane 16 (BGCC#463) lane 17 (*M. gordonae*), lane 18 (*M. szulgai*), lane 19 (*M. kansasii*), lane 20 (*M. terrae*), lane 21 (*M. malmonse*), lane 22 (*M. gastri*).

Table 3 PRA (PCR restriction analysis) of *rpoB* gene of mycobacterial strains isolated from water supply of some educational institutes in Jabalpur.

Lane	Isolate No.	Isolated from	No. of bands	Mol. Wt.	Strain identified
1	BGCC#458	Gun Carriage Factory School	1	270	<i>M. gordonae</i>
2	BGCC#460	Govindram Seksaria College	1	270	<i>M. gordonae</i>
3	BGCC#470	Holy Public School	1	270	<i>M. gordonae</i>
4	BGCC#455	Kendriya Vidyalaya of G.C.F No.1	2	115,200	<i>M. szulgai</i>
5	BGCC#456	Kendriya Vidyalaya C.O.D	2	115,200	<i>M. szulgai</i>
6	BGCC#457	Gun Carriage Factory School	2	115,200	<i>M. szulgai</i>
7	BGCC #462	Leonard High School	2	115,200	<i>M. szulgai</i>
8	BGCC#461	Nuvyug Arts and Commerce College	2	90,205	<i>M. kansasii</i>
9	BGCC#464	St.Aloysius Senior Secondary School	2	60,195	<i>M. terrae</i>
10	BGCC#465	M.G.M Higher Secondary School	2	60,195	<i>M. terrae</i>
11	BGCC#466	Sanatam Dharm Higher Secondary School	2	60,195	<i>M. terrae</i>
12	BGCC#467	Hitkarni Primary School	2	60,195	<i>M. terrae</i>
13	BGCC#468	St.Paul Higher Secondary School	2	195/60	<i>M. terrae</i>
14	BGCC#269	Shishu Shiksha Mandir School	2	60,195	<i>M. terrae</i>
15	BGCC#459	Government Science College	2	75.190	<i>M. malmonse</i>
16	BGCC#463	Leonard High School	2	105,180	<i>M. gastri</i>
17	Ref. strain	-----	1	270	<i>M. gordonae</i>
18	Ref. strain	-----	2	115,200	<i>M. szulgai</i>
19	Ref. strain	-----	2	90,205	<i>M. kansasii</i>
20	Ref. strain	-----	2	60,195	<i>M. terrae</i>
21	Ref. strain	-----	2	75,190	<i>M. malmonse</i>
22	Ref. strain	-----	2	105,180	<i>M. gastri</i>

as small tender, red-blue papulonodules, 0.5 to 1.5 cm in diameter with mamillated or ulcerated surfaces and with or without proximal lymphangitic spread in a sporotrichoid

pattern. The patient can have localized lymphadenitis but systemic signs of toxicity; fever, chills, malaise, nausea, and vomiting. The clinical differential diagnosis includes

sporotrichoid disease other than mycobacterioses, pyogenic infection, sarcoidosis, and other infectious granulomas³.

M. kansasii and *M. gordonae* both have been reported earlier from swimming pool water^{3, 8, 13, 26, 27} and *M. gordonae* alone has been reported from municipal water supply^{28, 29, 30}. During the present study *M. kansasii* has been isolated from water samples of Nuvyug Arts and Commerce College. Nontuberculous mycobacteria especially *M. kansasii* has got great significance in immunocompromised individual. It is worldwide in distribution but is especially prevalent in water samples. Disease is probably caused by inhalation of aerosolized organisms; skin infections are much less common. A cutaneous involvement occurs in normal or previously traumatized skin, and is commonly seen in patients with immunodeficiency. The infection incubation time of *M. kansasii* is 15 days to several months. Clinical manifestations include red-to-violaceous indurated papules or plaques; pustular, ulcerated, crusted or verrucous papules or nodules; cellulitis; abscesses; joint pain including arthritis, synovitis, and bursitis; cervical adenopathy and a sporotrichoid adenopathy of an involved extremity³¹.

During the present study, *M. malmoense* was isolated from samples of Government Science College. This organism is reported from municipal drinking water³². One isolate of *M. gastri* was isolated from St. Thomas High School samples during present investigation. This organism had also been reported from human gastric lavage specimen or sputum specimens as causal residents and is not considered etiologic agent of disease³³.

The species of mycobacteria found in present study are opportunistic pathogens except *M. terrae* and *M. gastri* and are very frequently isolated by many workers from various environmental samples especially water. The presence of these organisms in water sample might be attributed due to resistant to chlorine treatment due to formation of biofilms³⁴.

Mycobacteria are not normal flora of drinking water and other water samples and therefore, presence of mycobacteria in the drinking water is seldom analyzed. It is important to point out that many outbreaks of mycobacterial infections associated with exposure to medical solutions³⁵, industrial aerosols³⁶ and hot tubs and spas³⁷ have occurred inspite of disinfection of the possible sources. It is important to develop alternative strategies for reduction in number of NTM in the drinking water. One strategy for reduction of NTM is reduction of particulates turbidity in raw and treated water²⁹. Another approach would be to identify novel disinfectants that are active against NTM. Identification of factors leading to disinfectant resistance of NTM would greatly contribute to this goal.

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