Prevalence of mycobacteria in water reservoirs of Albaha, Saudi Arabia

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ABSTRACT

الأهداف : تحديد نسبة وجود بكتيريا المتفطرات (mycobacteria) في مياه السدود في منطقة الباحة بالمملكة العربية السعودية

الطريقة : قد أجريت دراسة وبائية خلال الفترة من 20 يونيو 2013 م حتى 30 يونيو 2013 م في منطقة الباحة، المملكة العربية السعودية جمعت 520 عينة (رمل وطين وحجارة ومواد نباتية متحللة) من 13 سداً من المنطقة . وكانت هذه السدود الثلاثة من مدينة العقيق (سد العقيق وثراد والمشيريف) و4 سدود من مدينة المندق (سد المظلمات والخرار والصدر ومدهاس) وسد واحد من مدينة الباحة (سد شهباء) و 5 سدود من مدينة بلجرشي (سد الظروة والعريشيتن وماطوة والهيجة والمرباة) . ثم تمت زراعة العينات من هذه السدود على وسط لونستاين ينسن (Lowenstein–Jensen) في حرارة 20 و30 درجة مئوية وتم التعرف على المتفطرات المعزولة عبر صفات نموها وتحاليل الإنزيمات والخصائص الكيميائية الحيوية وعبر دراسة جيناتها الرايبوسومية (16S rRNA) وأنماط جين اتش . أس . بي–65 (restriction fragment patterns of the hsp65 gene) .

النتائج : تم العثور على المتفطرات من 79% من عينات السدود المختلفة ووجد أنها تنتمي إلى 11 نوعاً (species) مختلفة .

الخاتمة : المتفطرات الموجودة في سدود منطقة الباحة بالمملكة العربية السعودية بنسبة عالية (79%) وبالتالي يجب أن يتم توخي الحذر من انتشارها إلى الإنسان والثروة الحيوانية بالمنطقة .

Objectives: To investigate for the presence of mycobacteria at water dams of Albaha, Saudi Arabia.

Methods: An epidemiological study was conducted between 20 - 30 June 2013 in the Albaha region, Kingdom of Saudi Arabia. Five hundred and twenty samples (sand, wet stones, clay, and decayed vegetation) were collected from 13 dams in the region. These locations were: 3 dams from Alaqiq city (Thrad, Alaqiq, and Almshereq); 4 dams from Almandaq city (Almudlamat, Alkhrar, Alsader, and Medhas); one dam from Albaha city (Shehba'a); and 5 dams from Baljerashi city (Alzarawah, Alareshaen, Almatwah, Alheajh, and Almarbah). Samples from these locations (n=520) were inoculated on Lowenstein-Jensen media. The isolated *Mycobacterium (M.)* obtained were identified by standard culture, enzymatic tests, biochemical characteristics, comparison of mycolic acid profiles, 16S rRNA gene sequencing, and restriction fragment patterns of the *hsp65* gene polymerase chain reaction product.

Results: *Mycobacterium* isolates were recovered from 79% of the samples obtained from all types of samples and locations. A total of 145 of the isolates were found to belong to the 11 *Mycobacterium* species: 5 *M. intracellulare*, 8 *M. abscessus*, 9 *M. szulgai*, 12 *M. fortuitum*, 12 *M. avium*, 14 *M. kansasii*, 15 *M. simiae*, 15 *M. gordonae*, 16 *M. terrae* complex, 18 *M. chelonae*, and 21 *M. malmoense*.

Conclusion: *Mycobacterium* species is present at high percentages in Albaha dams. The findings support a nationwide study to understand the clinical importance of environmental *Mycobacterium* in Saudi Arabia.

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Nontuberculous mycobacteria (NTM) are environmental, opportunistic human pathogens present in environmental sources like tap water, soil, domestic and wild animals, milk, and food products.[1] Primarily, NTM disease develops among immuno-compromised individuals, but recently it is emerging among immuno-competent individuals.[2] It can cause significant, and hard to treat skin and soft tissue infections,[3] and has been shown to cause an increasing quantity of pulmonary infections.[4] Soil and water has been found to contain *Mycobacterium (M.) fortuitum, M. gordonae, M. nonchromogenicum, M. terrae, M. chelonae,* and *M. malmoense*.[4-10] It has also been shown that mycobacteria are resident in some water distribution networks, and can cause significant human infections.[5-9,11] Alarmingly, NTM were also identified in some household tap and drinking water supply systems.[12-14] However, much less is known regarding its identification and prevalence in the Kingdom of Saudi Arabia (KSA). Studies have reported NTM infections from different regions of KSA,[15] but the real magnitude of the disease is still unknown. An important recent study[15] documents a high rate of 67.1% clinically relevant respiratory diseases caused by pulmonary NTM infections in KSA. Similarly, neighboring countries have reported an increasing prevalence of NTM diseases.[16,17]

So far, there is a lack of previous report available on the presence of NTM in water distribution systems, or water reservoirs in KSA, and the results presented here for an arid (desert) habitat, are of significant importance. Early reports have shown elsewhere that NTM is resident in drinking water distribution systems.[5,6,8,9,11] This study, therefore, aims to determine if NTM that can cause serious human diseases are present in the dams of Albaha region, which is used to provide potable water to the human population and for agriculture use.

Methods. This is an epidemiological study conducted between 20 - 30 June 2013 in the Albaha region, Kingdom of Saudi Arabia.

Literature search. A PubMed (Medline) and EMBASE web database search was carried out to cover the articles published using the key words: 'mycobacteria', 'nontuberculous' and 'mycobacterium'. Afterwards, articles were chosen and studied by reading their titles and abstracts. Finally, all related articles were retrieved and evaluated.

Ethical approval. Approval to carry out the research was obtained from the Institutional Review Board, Faculty of Applied Medical Sciences, Albaha University.

Sample collection. A total of 520 samples (130 samples per type) were collected from 13 locations in Albaha region. Ten samples per type per location were obtained. The sample types were: sand, wet stones, clay, and decayed vegetation. The 13 locations were: 3 dams from Alaqiq city (Thrad, Alaqiq, and Almshereq); 4 dams from Almandaq city (Almudlamat, Alkhrar, Alsader, and Medhas); one dam in Albaha city (Shehba'a), and 5 dams from Baljerashi city (Alzarawah, Alareshaen, Almatwah, Alheajh, and Almarbah). Samples were collected from different sites from each location. All were collected in the summer (June) of 2013. Samples were kept in sterile collection containers (SaudiPlast, Jeddah, KSA) used for fecal and urine collection placed on ice, and processed for isolation of mycobacteria within 8 hours. The water content of the samples was determined using Decagon Lite (AquaLab, Hopkins, Pullman, Washington, USA).

Isolation of mycobacteria. One gram of each sample was mixed with 5 ml of sterile normal saline and vortexed with one gram of sterile glass beads (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The resulting mixture was allowed to settle for 15 minutes. Finally, 4 ml of the resulting supernatant was collected and immediately mixed with 9 ml of 0.75% (w/v) hexadecylpyridinium chloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). This suspension was incubated at room temperature overnight, centrifuged (5000 g for 30 minutes) using a benchtop centrifuge (Hettich Lab Technology GmbH, Tuttlingen, Germany), and then the supernatant was emptied. The resulting pellet was resuspended in 0.5 ml of sterile Middlebrook 7H9 broth (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), then divided immediately into 2, 0.25 ml inocula, which was immediately distributed on 2 Lowenstein-Jensen (LJ) medium slopes (Oxoid, Hampshire, United Kingdom) (one incubated at 20°C, and the other at 30°C).

Identification of mycobacteria. Initially, standard culture, growth rates, colony morphology, pigmentation, enzymatic tests, and biochemical characteristics were used for the identification of *Mycobacterium* by comparison to known reference strains.[18] The method described by Goodfellow and Magee[18] was used for the identification of mycolic acid profiles of the mycobacteria isolated. Restriction enzyme digestion patterns of BstEII and HaeII (Thermo Scientific, KMG Trading, PAK Free Trade Zone, United Arab Emirates) of polymerase chain reaction (PCR) product of the 65-KDa *hsp65* heat shock protein gene were used as described previously,[19,20] and 16S rRNA gene sequencing was used to confirm the species. Sequencing was performed per the Allan Wilson Genome Service Centre (Massy University, Palmerston North, NZ). The 16S rRNA gene PCRs were carried out in a Hybaid PCR

express machine (Eppendrof AG, Hamburg, Germany). The PCR reaction mixes consisted of 0.5 U Taq DNA polymerase (Eppendorf AG, Hamburg, Germany), 5 ng of each primer (Invitrogen New Zealand Ltd., Auckland, NZ), 5 µl of 10x PCR buffer (Eppendorf), one µl of PCR nucleotide mix (Eppendorf), and the volume was made up to 50 µl with sterile Milli-Q (deionized water), and passed through a PCR reaction consisting of a denaturation step at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 47°C for 30 seconds, and completed with a final elongation step of 65°C for 2 minutes. The primers used were the *16sF*: 5'-CGA CTA HAG GGT GGT ATC TAA T-3,' and the *16sR*: 5'-AGA GTT TGA TCM TGG-3'.

Statistical analysis. To determine if the prevalence of mycobacteria in Albaha water is significantly different, *p*-values <0.05 was considered significant using the Z test for 2 population proportions. That is, the first being the percentage of NTM positive samples detected in this study, while the second was the percentage of NTM positive samples from Brisbane, Australia potable water.[14]

Results. Nontuberculous *Mycobacterium* species were recovered from every location and sample type. A total of 411 of the 520 samples (79%) were positive for NTM, yielding 645 isolates (**Table 1**) with actual colony counts obtained from the different samples on

LJ media (**Table 2**). Most of the isolates were recovered from samples whose moisture content ranged between 18-80%. Decayed vegetation samples were the most positive for NTM (89%), while wet stones were the least positive (70%). Alaqiq dams were the least positive for NTM compared with other locations, 72.5% compare to 79% or above in all other locations. Most isolates (334 of 645 isolates, 51.8%) were recovered from LJ slopes incubated at 20°C, whereas 48.2% were recovered at 30°C (311 of 645). All identified isolates were recovered at both temperatures. Compared with the prevalence of NTM detected in potable water in Brisbane, Australia (236 out of 384 samples were positive; 61.4%),[14] the results obtained in this study for the water reservoirs in Albaha, which are used for human consumption, recreation, and agriculture (411 out of 520 samples were positive; 79%) are significantly higher (Z-score: 5.7924; *p*=0.0).

Speciation of mycobacteria. Eleven species were identified from 145 of the 645 obtained isolated: 5 *M. intracellulare*, 8 *M. abscessus*, 9 *M. szulgai*, 12 *M. fortuitum*, 12 *M. avium*, 14 *M. kansasii*, 15 *M. simiae*, 15 *M. gordonae*, 16 *M. terrae* complex, 18 *M. chelonae*, and 21 *M. malmoense* (**Table 3**). The selected 145 isolates for identification were chosen as they represented different colony morphologies. The *M. kansasii* (n=14) isolates did not hydrolyze Tween 80

Table 1 - Numbers of positive samples per location and type in an epidemiological study conducted at Albaha region, Kingdom of Saudi Arabia.

Location	Sand	Wet stones	Clay	Decayed vegetation	Total
			n (%)		
Alaqiq city (3 dams)*	23 (77.0)	19 (63.0)	21 (70.0)	24 (80.0)	**87 (72.5)**
Almandaq (4 dams)	33 (82.5)	29 (72.5)	34 (85.0)	36 (90.0)	**132 (82.5)**
Albaha (one dam)	8 (80.0)	7 (70.0)	9 (85.0)	10 (100)	**34 (85.0)**
Baljerashi (5 dams)	39 (78.0)	36 (72.0)	37 (74.0)	46 (92.0)	**158 (79.0)**
Total	**103 (79.0)**	**91 (70.0)**	**101 (77.7)**	**116 (89.0)**	**411 (79.0)**
*For each dam, 10 samples were collected per sample type					

Table 2 - Colony counts obtained from the different samples on Lowenstein-Jensen media in an epidemiological study conducted at Albaha region, Kingdom of Saudi Arabia.

Location	Sand	Wet stones	Clay	Decayed vegetation	Total
Alaqiq city (3 dams)*	42 (10)	22 (6)	30 (6)	51 (12)	**145 (34)**
Almandaq (4 dams)	53 (13)	37 (9)	39 (9)	58 (13)	**187 (44)**
Albaha (1 dam)	17 (4)	8 (2)	11 (3)	19 (4)	**55 (13)**
Baljerashi (5 dams)	81 (17)	37 (9)	47 (10)	93 (18)	**258 (54)**
Total	**193**	**104**	**127**	**221**	**645 (145)**
No. subjected to identification	44	26	28	47	**145**
*For each dam 10 samples were collected per sample type. (Numbers between parentheses are those subjected to speciation [identification])					

Table 3 - Identified nontuberculous mycobacterial species per location and sample type in an epidemiological study conducted at Albaha region, Kingdom of Saudi Arabia.

Species	Sand	Wet stones	Clay	Decayed vegetation	Total
M. intracellulare	1[*]	0	3[‡,§]	1[†]	5
M. abscessus	1[†]	1[†]	2[†,‡]	4[*,‡,§]	8
M. szulgai	2[§]	0	3[†]	4[†,§]	9
M. fortuitum	3[†]	0	4[*,†]	5[*,†]	12
M. avium	3[*,§]	3[*,§]	2[*]	4[*,§]	12
M. kansasii	0	0	7[†,‡,§]	7[*,§]	14
M. simiae	4[‡,§]	2[‡]	3[B]	6[‡,§]	15
M. gordonae	7[†,‡,§]	0	5[*,†,§]	3[*,§]	15
M. terrae	0	0	5[‡,§]	11[*,†,‡,§]	16
M. chelonae	0	0	6[*,†,§]	12[*,†,‡,§]	18
M. malmoense	8[*,†,‡]	3[*,†]	3[†]	7[†,‡]	21
Total	29	9	43	64	145

M - Mycobacterium, [*]Alaqiq, [†]Almandaq, [‡]Albaha, [§]Baljerashi

and were photochromogenic, and produced mycolic acids I, III, and IV. The *M. terrae* isolates (n=18) were slow growing, non-pigmented, nitrate-reductase-negative, catalase-positive, hydrolyzed Tween 80, and produced mycolic acids I, and IV.[18] The *M. fortuitum* (n=12) isolates reduced nitrate, and produced mycolic acids I, II, and V.[18] The *M. chelonae* (n=18) isolates were rapidly growing, did not reduce nitrate, were all non-pigmented, and produced mycolic acids I and II.[18] Both *M. fortuitum* and *M. chelonae* isolates were catalase- and arylsulfatase-positive. The *M. malmoense* (n=21) isolates were slow growing catalase-positive, and produce mycolic acids I, II, and IV.[18] Fourteen of the 21 *M. malmoense* isolates hydrolyzed Tween 80. The *M. fortuitum*, *M. chelonae*, and *M. malmoense* were non-pigmented. All *M. gordonae* obtained were pigmented (n=15), slow growing, urease-negative, nitrate-reductase-negative, and catalase-positive. They also did not hydrolyze Tween 80, and produced type I, III, and IV mycolic acids.[18] The *M. intracellulare* (n=5) isolates were non-pigmented, did not hydrolyze Tween 80, were catalase- and arylsulfatase-positive, and produced mycolic acids I, IV, and V. The *M. abscessus* (n=8) isolates were non-chromogenic, rapidly growing, arylsulfatase-positive, nitrate reductase-negative, and produced mycolic acids I and II. The *M. szulgai* (n=9) were pigmented, slow growing, urease-, arylsulfatase- and nitrate reductase-positive, and produced mycolic acids I, III, and IV. The *M. simiae* isolates (n=15) were nitrate reductase-negative, did not hydrolyze Tween 80, and produced mycolic acids I, II, and IV. The

mycolic acid and restriction fragments produced from the amplified *hsp65* gene from the tested isolates were identical to that previously described.[18]

Discussion. Mycobacterial infections, as well as extra pulmonary NTM diseases in KSA are increasing rapidly,[21,22] however, little is known regarding NTM infections and its prevalence in the country.[23-25] The prevalence of NTM is expected to rise as immuno-compromised medical conditions (transplantations, genetic disorders, various immunosuppressive illness, and treatments) are escalating. A recent study reports an increasing problem of NTM infections among Saudis.[15] Most subjects in their study being Saudi nationals and male gender is in agreement with earlier studies showing that the origin of patient and gender are risk factors for NTM infections.[16,26] The findings of that study also suggest that Saudi nationals, particularly elderly men are at a relatively high risk for NTM infections in KSA.[15]

We report that pathogenic *Mycobacterium* are present in water reservoirs in KSA, which are used for human consumption, agriculture, and recreation. Nonetheless, environmental mycobacteria have been isolated from 72% of drinking water distribution systems in France,[8] 38% in the USA,[7] and 35-80% of water samples from the Finnish water distribution system.[27]

In our study, every type of sample and every location yielded mycobacteria, suggesting the high prevalence of environmental mycobacteria in water dams of Albaha region. Eleven different mycobacterial species recovered in our study indicates that the dams of Albaha region can support the growth of a wide variety of slow and fast growing mycobacterial species, and is not confined to a selected few species. Given the diversity of sample types and the different locations, we infer that mycobacterial species are not associated with a particular geographic location, or habitat. Isolation of mycobacteria from samples with low moisture content shows the ability of mycobacteria to survive in these conditions. The ability of mycobacterial bacterial cells to replicate in these conditions is unknown, but the survival and viability is confirmed. The *M. intracellulare* isolation from the dams of Albaha is alarming given that it is the main pathogen causing pulmonary diseases in many parts of the globe,[28] and it has also been detected in endoscopic duodenal biopsy findings of children in KSA.[29] The *M. intracellulare* and other NTM, has also been previously isolated from rainwater tanks,[30] soil, and house dust.[31,32]

The *M. kansasii*, another species isolated during this study was also found in potable water, and in an appendiceal abscess due to *M. kansasii* in a Saudi child

with AIDS.[33,34] A third species isolated during this study, *M. abscessus*, a saprophyte associated with water[35,36] caused several outbreaks, and many human infections affecting the lungs, skin, or soft tissues,[37-40] and chronic pulmonary lung disease in 2 immunocompetent patients in KSA.[24]

Certain study limitations should be acknowledged: it was designed for Albaha region only, and due to minimal representation of isolates to a single geographic region, it cannot be used to make national inferences; and it has no direct clinical implication. Nonetheless, the study sought to identify the prevalence of NTM in Albaha water bodies, and compared this to that discovered in Brisbane water in Australia.[14] The prevalence detected in this study are significantly higher (411 out of 520 samples were positive; 79%) (Z-score: 5.7924: p=0), than the results obtained from the Brisbane, Australia study (236 out of 384 samples were positive; 61.4%). Findings obtained in this study support an urgent need to study the implications and clinical significance of NTM in Albaha, which are present at a high rate in the region's water reservoirs used for human consumption, recreation, and agriculture.

In conclusion, this study indicated that NTM is prevalent in Albaha water reservoirs, and therefore, may be a risk factor for clinical infections. It can be concluded that prevention of pulmonary and extra-pulmonary NTM diseases in the Albaha community requires the authorities to address the NTM present in water reservoirs in the regions. Our findings emphasize the need to explore all the risk factors, including water human consumption, recreation, and agriculture use that many lead to NTM disease in KSA. Further, the findings suggest the need for a large-scale nationwide study to investigate the real magnitude of NTM prevalence in the country, and its association with clinical infections.

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