Isolation and identification of *Mycobacterium avium* complex and other nontuberculosis mycobacteria from drinking-water in Basra governorate, Iraq

A.A. Al-Sulami,[1] A.M.R. Al-Taee[2] and Q.H. Wida'a[1]

استفْراد وكشف معقَّد المتفطِّرات الطيرية وغيرها من المتفطرات غير السلية في مياه الشرب في محافظة البصرة في العراق

أمينَ عبد الجبار السُّلَمي، أسعد محمد رضا الطائي، قاسم حسن وداعة

الخلاصة: استهدفت هذه الدراسة التعرُّف على وجود معقَّد المتفطِّرات الطيرية وسائر المتفطرات غير السلية في مياه الشرب في محافظة البصرة في العراق، ومدى حساسيتها لمختلف المضادات الحيوية، وتأثير 0.5 مغ/ ل من الكلور على بقائها حية. وقد جمع الباحثون 404 عينة من مياه الشرب من ثلاث وثلاثين من مناطق المحافظة في الفترة من تشرين الثاني/ نوفمبر عام 2006 إلى آب/ أغسطس عام 2007 وحضن الباحثون العينات المرشَّحة لمدة لا تزيد على سبعة أيام في مستنبت ثنائي الطور – أحاديِّ الطور أُعِدَّ من مرق السل وآغار لوفنشتاين جنسن. وتعرّف الباحثون على 252 من المستفردات، على أن 21 منها لمعقد المتفطرات الطيرية، و15 للمتفطرات البحرية، و30 للمتفطرات الكنزاسية، و20 للمتفطرات النسناسية، و19 للمتفطرات الشغلائية، و16 للمتفطرات القيطمية، و11 للمتفطرات المالمونزية، و37 للمتفطرات التصادفية، و50 للمتفطرات القيحية، و33 للمتفطرات الـخُرَاجية. وأجريت اختبارات على المستفرَدات للتعرف على حساسيتها للمضادات الحيوية وعلى قدرتها على تحمُّل الكلور بتركيز 0.5 مغ/ ل. وخَلَصَ الباحثون إلى أن وجود هذه الجراثيم الـمُمْرِضَة في مياه الشرب، يجعل المياه غير صالحة للاستهلاك البشري.

ABSTRACT This study aimed to determine the occurrence of *Mycobacterium avium* complex and other nontuberculous mycobacteria in drinking-water in Basra governorate, Iraq and their susceptibility to several antibiotics and the effect of 0.5 mg/L of chlorine on their survival. A total of 404 samples of drinking-water were collected from 33 different districts of the governorate from November 2006 to August 2007. Filtered samples were incubated for 7 days or less in a monophasic–biphasic culture setup of tuberculosis broth and Lowenstein–Jensen agar. The 252 isolates were identified as *M. avium* complex (21), *M. marinum* (15), *M. kansasii* (30), *M. simiae* (20), *M. szulgai* (19), *M. xenopi* (16), *M. malmoense* (11), *M. fortuitum* (37), *M. chelonae* (50) and *M. abscessus* (33). Isolates were tested for antibiotic susceptibility as well as their ability to tolerate chlorine at a concentration of 0.5 mg/L. The presence of these pathogenic bacteria in drinking-water renders the water unfit for human consumption.

Isolement et identification du complexe *Mycobacterium avium* et de mycobactéries autres que celles responsables de la tuberculose dans l'eau de boisson du gouvernorat de Bassora (Iraq)

RÉSUMÉ La présente étude visait à déterminer l'occurrence du complexe *Mycobacterium avium* et de mycobactéries autres que celles responsables de la tuberculose dans l'eau de boisson du gouvernorat de Bassora (Iraq). Elle a aussi évalué leur sensibilité à plusieurs antibiotiques et l'effet de 0,5 mg/l de chlore sur leur survie. Au total, 404 échantillons d'eau de boisson ont été recueillis dans 33 districts différents du gouvernorat entre novembre 2006 et août 2007. Les échantillons filtrés ont été incubés pendant sept jours ou moins dans des milieux monophasique et biphasique comprenant un bouillon de bacilles tuberculeux et un milieu Löwenstein–Jensen. Les 252 isolats ont été identifiés comme étant le complexe *M. avium* (21), *M. marinum* (15), *M. kansasii* (30), *M. simiae* (20), *M. szulgai* (19), *M. xenopi* (16), *M. malmoense* (11), *M. fortuitum* (37), *M. chelonae* (50) et *M. bscessus* (33). Les isolats ont été analysés pour connaître leur sensibilité aux antibiotiques ainsi que leur capacité à tolérer du chlore à une concentration de 0,5 mg/l. La présence de ces bactéries pathogènes dans l'eau de boisson la rend impropre à la consommation humaine.

[1]Department of Biology, College of Education; [2]Department of Marine Environmental Chemistry, Marine Science Centre, University of Basra, Basra, Iraq (Correspondence to A.A. Al-Sulami: Aminabdulah@yahoo.com).

Received: 17/03/10; accepted: 22/07/10

Introduction

Mycobacterium avium complex (MAC) is one of the bacteria on the United States Environmental Protection Agency's (EPA) contaminant candidate list [1]. MAC and rapidly-growing mycobacteria such as *M. abscessus, M. mucogenicum, M. chelonae* and *M. fortuitum* can survive, persist, grow and colonize drinking-water supply systems [2–4].

When there is no evidence of person-to-person transmission, it can be assumed that humans are infected with nontuberculous mycobacteria from environmental sources [5]. However, several studies have failed to identify nontuberculous mycobacteria in water samples, often because of unsuitable isolation techniques [6]. Different growth rates, growth requirements and sources of water samples (e.g. treated, surface or natural) are all variables that affect the choice of method used for identification. Because of the slow growth of these organisms, pretreatment methods are necessary to limit bacterial and fungal overgrowth before mycobacteria can be detected. Unfortunately, the pretreatment method chosen may also prevent the detection of certain species of mycobacteria and reduce the rate of positive samples and the number of colonies seen [7].

The present study examined samples of drinking-water from different districts in Basra governorate, Iraq, to determine the occurrence of MAC and other nontuberculous mycobacteria, as well as their susceptibility to several antibiotics and the effect of 0.5 mg/L of chlorine on their survival.

Methods

A total of 404 samples of drinking-water were collected randomly for the purpose of this study from 33 districts in Basra governorate were collected during the period October 2006 to August 2007.

The concentration of residual chlorine for each sample was measured using a chlorine meter (Lovibond 2000).

Aliquots of 250 mL from each sample were filtered by membrane filtration technique using 47 mm cellulose acetate filters with a nominal pore size of 0.45 µm (Sartorius). The filters were cultured using the monophasic–biphasic culture setup method [8]. This was a simple system in which a slant of Lowenstein–Jensen agar (LJ) was prepared in test-tubes (160 mm size), followed by the introduction of 2 mL of tuberculosis (TB) broth to cover the lower portion of the slant only. The lower portion of the test-tube represents a biphasic environment (a liquid phase in contact with solid one), while the upper portion is single phase (solid only). To cultivate and isolate mycobacteria the filters were inoculated in TB broth for 5 min. with shaking, after which the TB broth medium was poured in the test-tubes containing the slant, mixed well and then tilted once or twice to cover the upper portion of the slant prior to incubation. The test-tubes were incubated aerobically at 37 °C for 7 days.

The suspected isolated mycobacteria were identified using a battery of biochemical tests according to the methods of Holt et al. [9] and Harley and Prescott [10]: oxidase test, catalase test, nitrate reduction test, tween 80 hydrolysis, growth with 5% NaCl, growth on MacConkey agar without crystal violet, urease test, pigmentation test, arylsulfatase test, pyrazinamidase test and H_2S production.

Mycobacteria isolates were also tested for their antibiotic susceptibility according to Piddock [11] by using 10 antibiotic disks: rifampin, kanamycin, streptomycin, azithromycin, ciprofloxacin, tobramycin, cephalothin, pyrazinamide, isoniazid, ethambutol.

To determine their resistance to chlorine the *Mycobacterium* spp. isolates were exposed to 0.5 mg/L concentrations of chlorine over 4 different time periods (30, 60, 90 and 120 s) [12].

The data were analysed using simple descriptive methods.

Results

Of the 200 samples taken in the winter season 80 (53%) were free of chlorine, compared with only 175 (87.5%) of the 200 summer samples.

A total of 252 isolates were identified as *Mycobacterium* spp., based on their morphological and biochemical characteristics in the battery of tests (Table 1). We succeeded in isolating slow- and rapid-growing bacteria from 30 districts in Basra. The slow-growing bacteria included: photochromogens, identified as *M. marinum, M. kansasii* and *M. simiae* (comprising 25.8% of total isolates), scotochromogens, identified as *M. szulgai* (7.5% of total isolates) and nonphotochromogens, which included *M. avium* complex, *M. xenopi* and *M. malmoense* (19.1% of total isolates). The rapid-growing bacteria included *M. abscessus, M. chelonae* and *M. fortuitum* and comprised 47.6% of total isolates.

Isolates of all the species were found in 30 districts of the governorate and during both winter and summer seasons at chlorine concentrations of 0–0.5 mg/L, but not at > 0.5–1.0 and > 1.0–2.0 mg/L.

Antibiotic susceptibility results using the tables supplied by the manufacturer of the kits showed that 7 species were sensitive to rifampin while the 3 rapid-growing bacteria species were resistant (Table 2). For kanamycin, erythromycin, cephalothin, tobramycin and streptomycin all species were sensitive or mildly sensitive. On the other hand *M. kansasii* and *M. marinum* showed resistance to pyrazinamide, while other species were sensitive or mildly sensitive. All species were sensitive to ciprofloxacin. *M. marinum* was resistant to isoniazid and *M. malmoense* was resistant to azithromycin.

Inactivation of *Mycobacterium* spp. isolates by chlorination showed that not all species of *Mycobacterium* were sensitive to chlorine at 0.5 mg/L as, with the exception of *M. szulgai,* log reduction did not exceed 1 even after 120s exposure (Table 3).

Table 1 Detection of *Mycobacterium* spp. in drinking-water samples from Basra governorate using a monophasic–biphasic culture setup

Mycobacterium spp.	*n*	Oxidase	Catalase	Pigmentation	Nitrate reduction	Tween 80 hydrolysis	Tellurate reduction	5% NaCl tolerance	H_2S production	Arylsulfatase	Pyrazinamidase test	Urease test	MacConkey agar w/out crystal violet
M. marinum	15	+	–	P	–	+	+	–	–	–	+	+	–
M. kansasii	30	+	+	P	+	+	+	–	–	–	–	+	–
M. simiae	20	+	+	P	–	–	+	–	–	–	+	+	+
M. szulgai	19	+	+	S	+	+	+	–	–	+	+	+	–
M. xenopi	16	+	–	–	–	–	+	–	–	+	+	–	–
M. avium complex	21	+	–	–	–	–	+	–	–	–	+	–	–
M. malmoense	11	+	–	–	–	+	+	–	–	–	+	–	–
M. fortuitum	37	+	+	–	+	+	+	+	+	+	+	–	+
M. chelonae	50	+	+	–	–	+	+	–	–	+	+	+	+
M. abscessus	33	+	+	–	–	+	+	+	–	+	+	+	+

n = no. of isolates; P = photochromogens; S = scotochromogens; + = positive; – = negative.

Table 2 Antibiotic resistance of *Mycobacterium* spp. isolated from drinking-water samples from Basra governorate

Mycobacterium spp. (*n* = 5 each)	% of isolates resistant to:									
	Rifampin (5 µg)	Kanamycin (30 µg)	Streptomycin (10 µg)	Ciprafloxacin (5 µg)	Tobramycin (10 µg)	Cephalothin (30 µg)	Pyrazinamide (6.6 µg)	Isoniazid (0.2 µg)	Ethambutol (5 µg)	Azithromycin (15 µg)
M. marinum	55	51	59	19	61	60	91	89	11	9
M. kansasii	55	50	10	3	9	12	95	11	0	51
M. simiae	55	12	11	6	9	0	0	53	12	22
M. szulgai	55	50	11	0	0	0	55	35	13	31
M. xenopi	55	57	21	31	57	60	54	35	0	0
M. avian complex	55	21	34	19	35	54	35	35	0	0
M. malmoense	38	23	21	39	0	56	55	35	0	85
M. fortuitum	92	51	53	22	25	55	25	55	49	53
M. chelonae	97	12	17	23	32	50	28	58	54	50
M. abscessus	95	32	53	12	15	55	16	68	59	53

n = no. of isolates.

Discussion

The data confirm that members of the genus *Mycobacterium* are present in the drinking-water of Basra and the rate of recovery of MAC was 8.3% in the 252 mycobacterial isolates. Other species of mycobacteria were recovered in high numbers and this may be related to the method of isolation used in this study, i.e. the monophasic–biphasic culture setup. The EPA reported that the culture and isolation of MAC bacteria from environmental samples is problematic because of their slow growth (it can take months to see colonies on plates), the particular nutrient requirements and the presence of other microorganisms that quickly outgrow MAC [1].

There is a consensus in mycobacterial laboratories that at least 1 of the media used should be a broth-based and that the use of solid media alone is not optimal and is too slow compared with the rapid turnaround time in broth. A study of Lowenstein-Jensen media versus 7H11 biplates demonstrated recovery of mycobacteria of 40% and 80% respectively on these media [13]. The authors concluded that decisions about which broth system to use will depend on the needs of each laboratory and the performance of each system within any particular laboratory. The BACTEC 460 system is still considered the gold standard among broth cultures, if one can contend with the problems associated with use of radioactive materials [14]. A BACTEC instrument was not available to us, however, and other methods were too expensive or inconvenient to perform in our laboratory [15].

In the present study we succeeded in isolating mycobacteria from drinking-water using monophasic–biphasic culture, a method which has previously been used only for isolation of *Mycobacterium tuberculosis* from clinical specimens [16]. The monophasic–biphasic culture method allowed us to reduce the period of incubation to 7 days (or less in some cases) compared with typically 21–28 days in Lowenstein-Jensen medium. The efficiency of this method may be related to the simultaneous use of 2 types of media (solid and liquid) and its versatility for culturing a wide variety of mycobacteria. Monophasic–biphasic culture is also inexpensive because of the small quantities of liquid and solid media consumed, and in view of our results we suggest that the method is suitable for the isolation of mycobacteria from drinking-water in developing countries.

Antibiotic drug resistance in *Mycobacterium* spp. is a worldwide problem [17]. A number of mechanisms for the resistance have been proposed, especially changes in the complex cell wall structure which contain mycolic acid and arabenogalactan in addition to peptidoglycan and which can reduce their permeability to antibiotics. For instance, isoniazid resistance has been associated with alterations in the *inhA* gene, which encodes an enzyme that functions in mycolic acid synthesis, a component of the mycobacterial cell wall [18].

Resistance of *Mycobacterium* spp. to chlorine has been attributed to the growth of bacterial cells in the form of a biofilm that allows these bacteria to acquire greater resistance to disinfectants than free cells [19.]. Our conclusion from this study is that the ability of *Mycobacterium* spp. to survive in distribution systems may depend on factors such as the efficiency of disinfection process, as indicated by the very low chlorine concentration of < 0.5 mg/L encountered in many districts of Basra.

The isolation of *Mycobacterium* spp. from drinking-water may depend on the techniques and materials used for culturing. It is noteworthy that *Mycobacterium* spp. were isolated for the first time from municipal drinking-water supplies in Basra and we attribute this to the monophasic–biphasic culture method use in this study.

Table 3 Inactivation in 0.5 mg/L chlorine of *Mycobacterium* spp. isolated from drinking-water samples from Basra governorate

Mycobacterium spp. (*n* = 5 each)	No. of $\log_{10}$ colonies present at different exposure times				
	Control	30 s	60 s	90 s	120 s
M. marinum	9.477	9.301	9.146	8.954	8.698
M. kansasii	9.462	9.301	9.146	8.954	8.602
M. simiae	9.301	9.176	9.000	8.778	8.477
M. szulgai	9.477	9.397	9.301	8.176	8.079
M. xenopi	9.414	9.255	9.113	8.903	8.477
M. avium complex	9.477	9.301	9.079	8.845	8.477
M. fortuitum	9.477	9.342	9.204	9.000	8.845
M. chelonae	9.431	9.301	9.146	8.954	8.968
M. abscessus	9.477	9.301	9.179	9.000	8.968
M. malmoense	9.477	9.301	9.176	9.000	8.968

n = no. of isolates.

References

1. Regulatory determinations for priority contaminants on the second drinking-water contaminant candidate list. Washington DC, Environmental Protection Agency, 2008 (EPA report 815-R-08-012).
2. Williams MM et al. Structural analysis of biofilm formation by rapidly and slowly growing nontuberculous mycobacteria. *Applied and Environmental Microbiology*, 2009, 75:2091–2098.
3. Falkinham JD 3rd et al. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking-water distribution system. *Applied and Environmental Microbiology*, 2001, 67:1225–1231.
4. Torvinen E et al. Survival of *Mycobacterium avium* in drinking-water biofilms as affected by water flow velocity, availability of phosphorus, and temperature. *Applied and Environmental Microbiology*, 2007, 73:6201–6207.
5. Covert TC et al. Occurrence of nontuberculous mycobacteria in environmental samples. *Applied and Environmental Microbiology*, 1999, 65:2492–2496.
6. Ford TT, Vincent V. Analytical methods for the detection of waterborne and environmental pathogenic mycobacteria. In: Pedly S et al. eds. *Pathogenic mythobacteria in water, a guide to public health consequences, monitoring and management*. London, IWA Publishing, 2004:55–73.
7. Thomson R et al. Comparison of methods for processing drinking-water samples for the isolation of *Mycobacterium avium* and *Mycobacterium intracellulare*. *Applied and Environmental Microbiology*, 2008, 74:3094–3098.
8. Al-Sulami A et al. A single step method for rapid isolation and identification of Mycoplasma pneumonia. *Eastern Mediterranean Health Journal,* 2002, 8:157–163.
9. Holt JG et al. *Bergey's manual of determinative bacteriology*. Baltimore, Williams and Wilkins, 1994:705–718.
10. Harley JP, Prescott LM. *Laboratory exercises in microbiology*, 3rd ed. Boston, McGraw-Hill, 1996:484.
11. Piddock LJJ. A review techniques use for the determination of antimicrobial resistance and sensitivity in bacteria. *Applied Bacteriology*, 1990, 68:307–318.
12. Johnson CH et al. Inactivation of *Helicobacter pylori* by chlorination. *Applied and Environmental Microbiology*, 1997, 63:4969–4970.
13. Wilson ML et al. Comparison of recovery rates for mycobacteria from BACTEC 12B vials, Middlebrook 7H-11 selective biplates and Lowenstein-Jensen slants in a public health mycobacteriology laboratory. *Journal of Clinical Microbiology*, 1995, 33:2516–2518.
14. Sewell DL et al. Comparison of the Septi-Chek AFB and BACTEC systems and conventional culture for recovery of mycobacteria. *Journal of Clinical Microbiology*, 1993, 31:2689–2691.
15. Flournoy D, Jeann JT. Modified Middlebrook 7H9 broth of the rapid detection of mycobacteria. *Clinical Laboratory Science*, 2001, 14:85.
16. Al-Sulami A et al. Rapid isolation and identification of *Mycobacterium tuberculosis* from pulmonary tuberculosis patients. *Asian Pacific Journal of Tropical Medicine*, 2009, 2:70–73.
17. Raviglione A et al. Global epidemiology of tuberculosis. *Journal of Clinical Microbiology*, 1995, 32:110–114.
18. Brook GF et al. *Medical microbiology*, 21st ed. Lebanon, Typo Press, 1998.
19. Lechevallier MW, McFeters GA. Microbiology of activated carbon. In: McFeters G.A ed. *Drinking-water microbiology, progress and recent developments.* New York, Springer Verlag, 1988:104–119.