A Comparison of Methods for Processing Drinking Water Samples for the Isolation of *Mycobacterium Avium* and *intracellulare*.

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Abstract

Several protocols for isolation of mycobacteria from water exist but there is no established standard method. This study compared methods of processing potable water samples for the isolation of *M.avium* and *M.intracellulare* using spiked sterilized water and tap water decontaminated using Cetylpyridinium Chloride (CPC) 0.005%. Samples were concentrated by centrifugation or filtration, and inoculated onto Middlebrook 7H10 and 7H11 plates, Lowenstein Jensen(LJ) slopes and into Mycobacterial Growth Indicator tubes (MGIT) ± PANTA (polymyxin, azlocillin, nalidixic acid, trimethoprim, amphotericin B). The solid media were incubated at 32º, 35º and 35ºC with CO_2 and read weekly. Results suggest filtration of water for the isolation of mycobacteria is a more sensitive method of concentration than centrifugation. The addition of Sodium Thiosulphate may not be necessary and may reduce the yield. M7H10 or 7H11 were equally sensitive culture media. CPC decontamination, whilst effective in reducing growth of contaminants

also significantly reduces mycobacterial numbers. There was no difference at 3 weeks between the different incubation temperatures.

INTRODUCTION

The isolation of Mycobacteria from both environmental and treated drinking water samples was first reported in the early 1900s. However it has only been in the last 3-4 decades that these environmental mycobacteria have been recognized as pathogens of human disease. As compared to *Mycobacterium tuberculosis*, these organisms are generally of low virulence and require a host defect for the establishment of disease (e.g. disseminated disease in AIDS patients, pulmonary disease in patients with underlying structural lung disease). However there is a subset of patients who develop pulmonary disease without an obvious immune defect or one that is yet to be defined. Nontuberculous Mycobacteria (NTM) have been demonstrated in drinking water(1, 7, 11, 12, 15, 16, 18, 26, 35), drinking water distributions systems(17, 18, 23, 32-34), hot water systems(4), spas(6) and pools(14, 19). However several authors have failed to identify NTM in water samples, often because of unsuitable isolation techniques. Variable growth rates, specific growth requirements and different sources

of water samples (eg treated/surface/natural) are all variables that will affect the choice of method for identification. Because of their slow growth, pre-treatment methods are necessary to limit bacterial and fungal overgrowth and hence detect mycobacteria. However, the pretreatment method chosen may also prevent the detection of certain species of mycobacteria, reducing the rate of positive samples and number of colonies seen. A number of different protocols have been described(29) but a standard protocol has not yet been established. Du Moulin and Stottmeier(5) first described the use of Cetylpyridinium chloride in 1978. They applied 0.04% to 1L samples of distilled water seeded with dilutions of 5 day old cultures of mycobacteria grown in M7H9 broth and allowed them to stand for 24 hours, prior to filtration, rinsing and applying membrane to M7H10 agar plates. Plates were incubated at 37°C (5-10%CO_2) for 60 days. A control group of samples were processed the same way but without CPC treatment. Survival of mycobacteria in spiked specimens varied from 1 to 100%, depending on the species - *M.kansasii* 18.4%, *M.gordonae 8.4%, M.intracellulare* 100%, *M.fortuitum* 1.1% and *M.bovis* 39.9%. These authors actually reported greater survival of *M.intracellulare* in treated (7400 viable units (vu)/L) vs untreated samples (440 vu/L). Schulz-Robbecke et al(27) compared Cetylpyridinium chloride (CPC), sodium hydroxide (NaOH) and formaldehyde (HCHO) for their efficacy as decontamination substances

for the isolation of mycobacteria from drinking water samples. They found that 0.005% CPC had the highest recovery of mycobacteria and the lowest contamination rates, using both spiked samples and environmental samples. This finding was confirmed by Neumann et al(22). Glover et al(10) found that 0.04%CPC decontamination of tap water samples resulted in less contamination than 1-3%NaOH or 5% OA, but also the highest number of samples with no growth. CPC was applied at this concentration for 24 hours to sterile water seeded with MAC to a final concentration of $1.5X10^3$ CFU/500ml. This resulted in a reduction of 89% in viable mycobacteria. NaOH 1% and OA 5% resulted in reductions of 64% and 59% respectively. Le Dantec(18) used membrane filtration followed by decontamination with Na dodecyl sulfate and NaOH, adjusting pH with 40% phosphoric acid. Using *M.gordonae* spiked sterile tap water they showed that this decontamination method reduced mycobacterial numbers to 1% of the original number.

Falkinham(7, 8) has suggested that for drinking water decontamination may not be required. In his study published in 2001(7) he processed samples initially without decontamination but if plates were overgrown, reprocessed them using CPC. Unfortunately it is not stated in the paper how often decontamination was necessary. Only 15% samples grew slow

growing mycobacteria (3% *M.avium*, 1% *M.intracellulare*) and there were 2% rapid growers.

Other variables that may affect the yield of mycobacteria from environmental water samples include sample volume, the use of Sodium Thiosulphate to neutralize chlorine based disinfectants, method of concentration (eg filtration vs centrifugation), culture media and incubation temperature.

In Queensland the main mycobacterial pathogen associated with pulmonary disease is *M.intracellulare* followed by *M.avium*, *M.abscessus* and *M.kansasii*. It has been postulated that patients acquire disease by inhaling aerosols containing mycobacteria from environmental water sources and water outlets in their homes(20). Patients may also aspirate contaminated water as a result of swallowing disorders or severe gastro-esophageal reflux disease(31).

This pilot study was undertaken to try and identify the best method of processing water samples for the isolation of mycobacteria prior to a larger environmental survey. The aim of this study was to compare different methods of processing drinking water samples for the isolation of species of mycobacteria pathogenic to humans, particularly

M.intracellulare and *M.avium,* with regard to concentration (centrifugation vs filtration), culture media (LJ, 7H10, 7H11, MGIT and MGIT+PANTA), and incubation temperature (32ºC, 35ºC, and 35ºC+CO2).

METHODS

M. avium (ATCC 35765) and *M. intracellulare* (ATCC 13950) were inoculated in 7H9 broth (0.5 McFarland), correlating to concentration $1.5x10^8$ CFU/ml and diluted to a concentration of 100 CFU/500ml water.

Control samples.

Organisms (*M.avium* and *M.intracellulare* separately) were added to 8x 500ml samples of sterile water (sterilized by filtration to preserve chlorination using MediaKap-2 - Hollow Fibre Media Filter 0.2µm - Spectrum Laboratories Inc.) to a final concentration of 100CFU/500ml. Sodium Thiosulphate (0.5ml of 10% solution) was added to 4 of the samples (2 *M.avium* and 2 *M.intracellulare*). Half of the samples were processed by filtration and half processed by centrifugation. (**Figure 1**)

Filtration was performed through 0.45µm cellulose nitrate filters (Sartorius AG 37070 Goettingen, Germany). Filters were then rinsed with 2ml sterile distilled water (SDW) and macerated in 3 ml SDW. From this 3ml, 0.1ml aliquots were then transferred in triplicate to LJ slants, M7H10 and M7H11 plates , sealed in gas permeable plastic bags for incubation at 32°, 35°

and 35°C +CO2. 0.5 ml aliquots were transferred to 2 MGIT tubes, 1 containing PANTA (polymixin, azlocillin, nalidixic acid, trimethoprim, amphotericin B).

Centrifugation: Four - 500ml samples (2 containing Na Thiosulphate -1 *M.avium, 1 M. intracellulare*) were centrifuged in 250ml sterile bottles at 5000gx 20min at 25 °C. The pelleted cells were rinsed twice with Phosphate Buffered Saline (PBS)(21). The resulting suspension was added to sterile diluent to make 3ml and 0.1ml aliquots were used to inoculate in triplicate each of the following: LJ slants, M7H10 and M7H11 plates. Plates were sealed in gas permeable plastic bags and incubated as previously indicated. Additional 0.5 ml aliquots were used to inoculate 2 MGIT tubes, with and without PANTA.

Tap water.

Tap water samples (4x 500ml) were collected after flushing for 2 minutes from a single tap within the laboratory. These tap water samples were spiked with *M.avium* (x2) and *M.intracellulare* (x2) to give a final concentration of 100CFU/500ml. Samples were then decontaminated with 0.005% CPC and incubated at room temperature for 30 minutes. Two samples (1 *M.avium*, 1 *M.intra*cellulare) were then processed by filtration and 2 processed by centrifugation, as described for sterile samples.

All plates were read weekly. At three weeks all plates were photographed digitally and colonies counted. Colonies from plates demonstrating growth were stained to confirm acid fast bacilli, and morphologically different colonies were subcultured on M7H10 agar and incubated at 35℃. Subcultured organisms were then identified to the species level using multiplex PCR as described by Wilton and Cousins(36). All colonies grown from the tap water samples were treated similarly.

Data were analyzed using SPSS v12.0 for Windows 2003 (Apache Software Foundation). Tests of association were performed using Fishers exact test for Chi Squared 2x2 tables. Statistical significance was defined as a 2 sided p value<0.05. Colony counts were also compared using the Mann Whitney U test as the values were not normally distributed.

RESULTS

There were 88 spiked sterile water cultures, and 44 spiked tap water samples. 83.3% of all filtered samples grew mycobacteria compared to 12.1% of all centrifuged samples (p<0.0001).

Of spiked sterile samples not treated with Sodium Thiosulphate, 52.3% grew mycobacteria compared to 43.2% of samples that were treated (p=0.223). For filtered samples the addition of Sodium Thiosulphate did not affect recovery. However, for centrifuged samples, 4.5% of treated samples were positive compared to 22.7% of untreated samples (p=0.058). Colony counts were lower in filtered sterile samples with Sodium Thiosulphate added (mean±SD: 151.7±169.8 vs 259.o±352.8 CFU/L; however this was not statistically significant (p=0.178) Mann Whitney U test (p=0.709) **(Figure 2)**.

There was no overall difference between Middlebrook 7H10 and 7H11 with 12 and 13 of 18 filtered samples showing positive growth respectively after 1 week. The LJ slants initially appeared less sensitive, but there was no difference between it and the Middlebrook media at 3 weeks (**Table 1**). There was no difference overall between the different incubation temperatures (**Table 2**).

For filtered samples CPC decontamination did not appear to affect the number of positive cultures at 3 weeks - 86.4% of filtered samples treated with CPC were positive at final reading, compared to 81.8% of those untreated. However colony counts were significantly reduced in spiked tap water samples (Mean±SD CFU/L 7.4±8.5) compared to sterile samples

(205.4±262.4; p=0.0001). This equates to a mean reduction to 3.6% of original numbers. (**Figure 3**)

At 3 weeks, 3 samples not treated with CPC were overgrown compared to none of those treated. Nine of 88 (10.2%) spiked sterile samples grew contaminants in addition to mycobacteria compared to 13/44 (29.5%) tap water samples. (p=0.012). These contaminants did not affect the ability to isolate mycobacteria. Of the spiked sterile samples, in 2 of these the plates had fungal overgrowth at week 4 – a week after they had been photographed, and this was likely aerial contamination when the plates were inspected for photography. Of the remaining 7 - 4 plates had single non-buff colonies, 2 had 2 colonies and 1 had 3. Whilst these were not formally identified it is presumed they entered the system during the processing of samples.

A number of samples grew morphologically different colonies on Middlebrook plates. These were subcultured and then identified to the species level using multiplex PCR and found to be the same organism. PFGE was not performed on these isolates. All colonies grown from the tap water samples were similarly processed. No other mycobacteria (other than the spiked organisms) were identified from the tap water samples.

DISCUSSION.

In this study we have demonstrated that filtration is a more effective method than centrifugation for isolating mycobacteria from water samples. Apart from having a far greater yield it was also simpler and more time efficient. To our knowledge there have been no published direct comparative studies, but previous authors have been able to isolate mycobacteria from water samples processed by centrifugation. Perhaps it was our technique, but alternatively the success of previous authors maybe related to much higher concentrations of mycobacteria in the water being sampled. In this study low concentrations of target organisms were used as may be expected to exist in suburban, treated, water supplies(3, 7, 9, 12, 18).

The majority of reported studies have processed samples with Sodium Thiosulphate to neutralize residual chlorine(2). It is not known whether neutralizing residual chlorine interferes with the ability to isolate mycobacteria by increasing bacterial overgrowth, or whether the presence of residual chlorine reduces the yield and diversity of species of mycobacteria subsequently isolated. As most opportunistic pathogenic NTM are relatively resistant to chlorine(18, 24, 25, 28, 30) the addition of

Sodium Thiosulfate may not be necessary and may increase contamination rates.

The Thiosulfate anion characteristically reacts with dilute acids to produce sulfur, sulfur dioxide and water: $S_2O_3^{2-}(aq) + 2H^+ (aq) \rightarrow S(s) + SO_2(g) + H_2O(l)$. Thiosulfate reduces the hypochlorite and in so doing becomes oxidized to sulfate. The complete reaction is: $4NaClO + Na_2S_2O_3 + 2NaOH \rightarrow 4NaCl + 2Na_2SO_4 + H_2O$.(13)

From our results it would appear that Sodium Thiosulphate may have some antibacterial properties in water, perhaps by generation of sulfur, as contamination rates and mycobacterial colony counts were less in those treated samples. Though not statistically significant, this is an interesting observation. Of importance, it would seem that for the purposes of isolating mycobacteria from water, the addition of Sodium Thiosulphate is unnecessary.

The addition of CPC to tap water samples spiked with *M.avium* and *M.intracellulare* resulted in 3.6% survival of organisms, but did not affect the number of positive samples using this concentration of organisms. The organisms used in our study were grown in 7H9 broth. It has been shown that antecedent growth conditions may affect susceptibility to chlorine based disinfectants. Water grown strains of *M.avium* were shown to be

significantly more chlorine resistant than those grown in medium(30). The magnitude of reduction of growth we have shown may not necessarily apply to water grown organisms from environmental or distribution system samples.

There were no differences between the temperatures tested nor between the different solid media overall. However the Middlebrook media were more sensitive at one week and provided the advantage of quantitation of growth over LJ. The MGIT system has recently been introduced for the culture of clinical specimens, and has not been used widely for the processing of water samples. Supplementation with PANTA (Polymyxin, Amphotericin B, Nalidixic acid, Trimethroprim and Azlocillin) is used to reduce contamination. A further study utilizing raw tap water samples (ie no decontamination) and the MGIT system (+PANTA to control contamination) is currently underway. The MGIT without PANTA used in this study did not contain OADC enrichment which may explain the lower yield using this system.

There have been a number of studies published using different methods to isolate mycobacteria from water samples, and no established standard. We have demonstrated that SodiumThiosulphate may not be necessary and may interfere with growth. We have confirmed the findings of

previous authors that CPC controls contamination, but significantly reduces mycobacterial growth also. Whilst it would be appealing to process samples without decontamination, the utility of this method would depend on the origin of the samples.

This study has added refinement to concentration and culture techniques for the isolation of mycobacteria from water; however the major challenge remains the need for decontamination to reduce bacterial and fungal overgrowth. We and others have demonstrated that the addition of CPC is effective for this purpose; however we have quantified the reduction in yield of *M.intracellulare* and *M.avium*, two of the main pathogens associated with lung disease and found it is significant. Given that that major environmental niche for *M.intracellulare* is in biofilms(7) and only small numbers are found in water samples, the detection of low concentrations of organisms is important. Perhaps a metagenomic study may obviate the need for any decontamination and culture method, and developments in this area are awaited with interest.

299 **Table 1. Positive cultures for mycobacteria after concentration by filtration**

300 **using different culture media after 1 week and 6 weeks (n=66).**

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Culture media	Week 1			Final Culture Result		
	Contaminated	Negative	Positive	Overgrown	Negative	Positive
7H10 (n=18)	1	5	12	0	2	16
7H11 (n=18)	0	5	13	1	0	17
LJ (n=18)	2	16	0	0	1	17
MGIT (n=6)	0	6	0	0	5	1
MGIT+PANTA (n=6)	0	3	3	0	2	4

Table 2. Comparison of incubation temperatures for culture of mycobacteria in both spiked sterile and tap water samples processed by centrifugation or filtration.

Concentration	Incubation Temperature				Total
	32°C	35°C	35°C+CO_2	BACTEC	
Centrifugation					
Contaminated	2	2	3	0	7
Negative	14	14	12	11	51
Positive	2	2	3	1	8
Total	18	18	18	12	66
Filtration					
Contaminated	0	0	1	0	1
Negative	0	0	0	7	7
Positive	18	18	17	5	58
Total	18	18	18	12	66

309 **Figure 1. Flow chart for processing of sterile water samples**

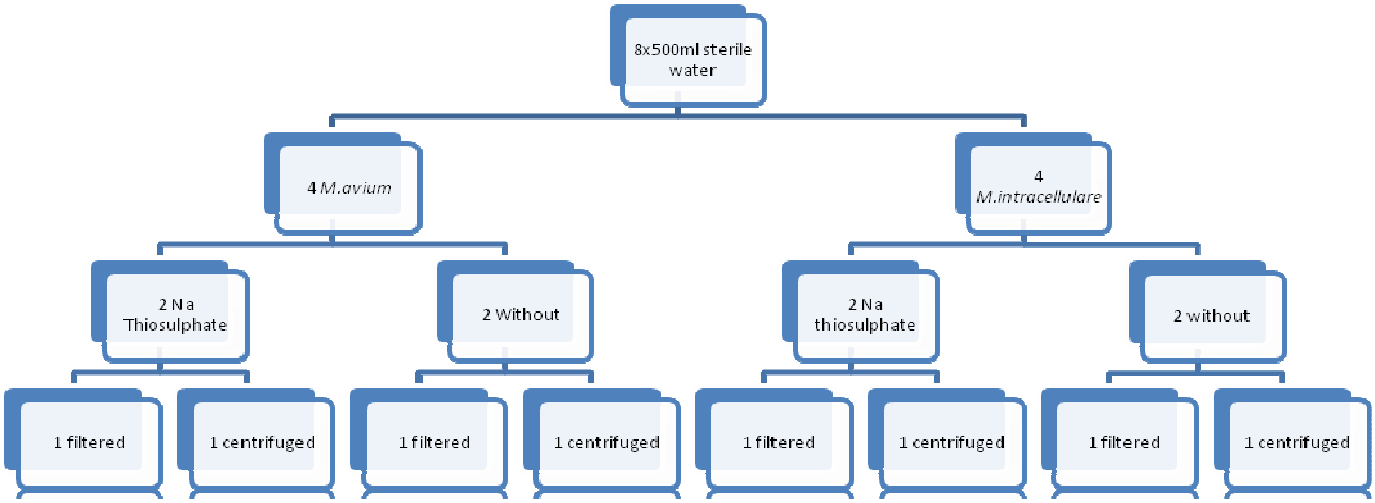

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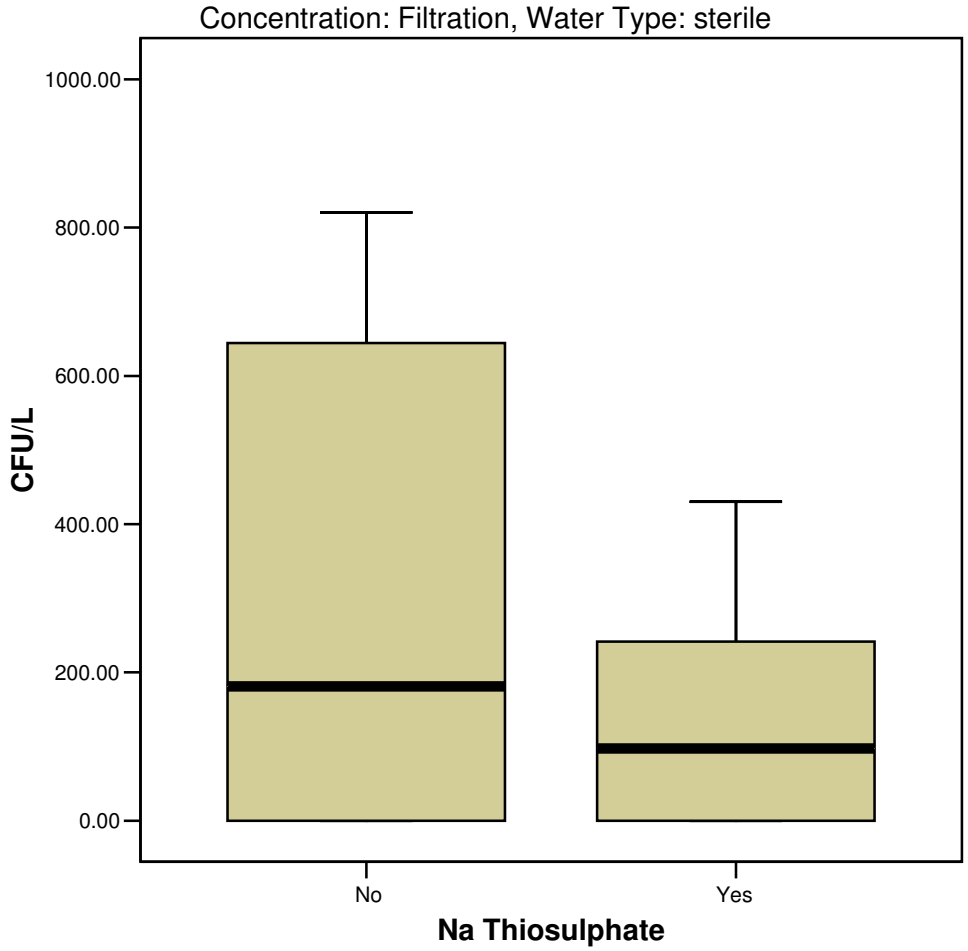
Figure 2. Boxplot demonstrating Median CFU/L (black bar), middle 2 quartiles (box) and range (extent of bar) of CFU/L Mycobacteria in spiked sterile water concentrated by filtration processed with and without the addition of NaThiosulphate.



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