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Version of Record: https://www.sciencedirect.com/science/article/pii/S266699192100107X
Manuscript_384dcde134980ef0e7ff6b76bb5b846a

Improved detection of non-tuberculous mycobacteria in hospital water samples

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Abstract

Non-Tuberculous Mycobacteria (NTM) are opportunistic pathogens commonly colonizing hospital water systems, and may be responsible for healthcare-associated infections (HAI). Investigation of HAI and outbreaks caused by NTM necessitates water analyses. However, NTM are slow-growing bacteria within the mesophilic community present in water, and they are difficult to detect. Prior to culture on specific media, their recovery usually requires decontamination and concentration steps. We assessed the effectiveness of filtration as regards the recovery of 7 NTM species in hospital water samples. We also compared the use of cetylpyridinium chloride (CPC) at different concentrations and Sodium Hydroxide (NaOH) in decontamination of water samples with mesophilic bacteria. Our laboratory protocol showed that membrane filtration was suitable for concentration and recovery of NTM from water. Sample decontamination with CPC was more NTM-preservative than NaOH. A combination of CPC at 0.005% and filtration allowed detection of NTM at low concentrations, ranging from 3 to 98 CFU/100mL according to the NTM species.

Key words: *Mycobacterium chimaera*; cetylpyridinium chloride; decontamination; water safety; healthcare-associated infections

Introduction

Non-tuberculous Mycobacteria (NTM) are opportunistic pathogens colonizing hospital water networks responsible for healthcare-associated infections (HAI) [1]. Environmental investigations are crucial as means of identifying sources of contamination and of improving prevention and control practices [1]. The worldwide outbreak of *Mycobacterium chimaera* surgical site infections has underlined the need for a standardized protocol to detect NTM in water in at-risk healthcare settings [2,3]. The detection of NTM in water containing fast-growing mesophilic bacteria requires decontamination, a procedure that may also inhibit NTM growth [4,5]. In cases of *M. chimaera* outbreak in cardiac surgery, the European Centre for Disease Prevention and Control recommended cetylpyridium chloride (CPC) as a means of decontaminating water samples, the objective being to

isolate NTM [6]. That said, the standard method for sputum sample decontamination uses sodium hydroxide (NaOH)[7]. A CPC decontamination detection limit is required for reliable interpretation of analyses. The aim of this study was to assess a method combining filtration and CPC decontamination as means of detecting NTM in water samples, in 7 species of NTM. The limit of detection was determined, and CPC was compared to NaOH as means of decontamination.

Material and Methods

NTM strains

M. chimaera (CIP107892T); *Mycobacterium smegmatis* (CIP7326); *Mycobacterium abscessus* (strain LDE1), isolated in feed water of washer-disinfectors of the endoscopy unit of Montpellier University Hospital; *Mycobacterium wolinskyi* (strain P2329), isolated from a cardiac surgical site infection [8]; *Mycobacterium chelonae* (strain MJH3) from the water tank of a heater-cooler unit for extracorporeal circulation; *Mycobacterium mucogenicum* (strain MJN1) and *Mycobacterium llatzerense* (strain MJN3-3) both of them isolated from tap water of the cardiac intensive care unit of Montpellier University Hospital [8].

Water sample treatment and culture conditions

Water samples were filtered through 0.45µm-porosity cellulose nitrate membranes (Sartorius) and then plated onto Middlebrook 7H10 agar media (Becton Dickinson) and incubated at 30°C fir 10 days (2 weeks for *M. chimaera*).

NTM recovery after filtration, CPC or NaOH treatment

For each NTM strain, a suspension of approximately 10^3 colony-forming units (CFU)/100mL was prepared in sterile water (Versylene Fresenius). Concentrations in NTM were determined by plating 100µL of suspensions onto media. One mL of NTM suspensions was treated for 30 min by either 1mL of CPC solution at 0.005% (m/V)(Sigma Aldrich), or 1mL of NaOH 4% (m/V) and then neutralized by

48mL of Phosphate-Buffered Saline. Bacteria were concentrated by filtration and the filter washed by 100mL of sterile water before culture. As a control, 1mL of suspension was filtered and cultivated without CPC or NaOH treatment. Filtration recovery rate corresponds to the mean ratio between the CFU numbers after filtration without CPC treatment and the initial CFU numbers; CPC survival rate corresponds to the mean ratio between CFU numbers recovered with and without CPC decontamination.

Detection limit of NTM in artificial tap water after CPC decontamination and filtration

The limit of detection after CPC decontamination was assessed on NTM suspension prepared in artificially contaminated water. This artificial water was prepared with 0.45µm-filtered tap water inoculated with approximately 10^3 bacteria/100mL of 5 mesophilic hydric bacteria isolated from the water network of Montpellier University Hospital: *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Sphingomonas paucimobilis*, *Methylobacterium sp.*, *Pseudomonas stutzeri*. The exact CFU numbers were determined after filtration of 5mL of artificial tap water and culture of the membrane onto Plate Count Agar medium (bioMérieux) at 30°C for 3 days.

NTM suspensions ranging from 1 to 2.5 10^3 CFU/100mL were prepared in artificial tap water. Five and 50mL of every suspension were cultivated before and after CPC treatment at 0.005% (m/V). Detection limit was defined as the lowest NTM concentration allowing positive detection. Experimentations were repeated twice.

Statistical analysis

Comparison of mean recovery and survival rates between each other and to the targeted value of 100% were performed with Student t-test. Gaussian distribution and equality of the variances we verified by Kolmogorov-Smirnov and Fisher tests using Instat®Version3.0 (GraphPad Software, Inc. San Diego, CA, USA).

Results and Discussion

Filtration is efficient as a means of concentrating NTM in water

For the seven species, mean filtration recovery rates ranged from 81.31% for *M. mucogenicum* to 100% for *M. abscessus*, and did not significantly diverge from 100% ($p<0.05$). While centrifugation or filtration is conventionally used for concentration of NTM in water samples, filtration appears more efficient [9]. Indeed, our results confirm that filtration is an effective means of recovery of NTM in water. Instead of discharging the membrane of filtration in a buffer before cultivating NTM [9], we directly plated it onto appropriate culture media in order i) to simplify the manipulation and adapt it to routine protocol ii) to avoid potential NTM loss induced by membrane discharge. Furthermore, filtration is more convenient than centrifugation as a means of concentrating bacteria from large volumes of water [3] and thereby enhancing the NTM detection limit.

CPC and NaOH survival rates

The main concern during detection of NTM in water is how to eliminate mesophilic bacteria while preserving NTM. In this study, tolerance to CPC decontamination varied according to NTM species. NTM survival rates after CPC ranged from 10.84% for *M. wolinskyi* to 100% for *M. abscessus* (Figure 1). For *M. abscessus* ($p=0.2263$) and *M. chimaera* ($p=0.2138$), the CPC survival rate did not differ significantly from 100%. NaOH 4% is usually recommended as a means of decontaminating and selecting mycobacteria in sputum samples not only in clinical microbiology, but also in water samples in environmental microbiology [10]. In our study, all NTM were more susceptible to NaOH than to CPC ($p<0.05$) (Figure 1). *M. abscessus*, *M. smegmatis*, *M. chelonae*, *M. wolinskyi* and *M. llatzerense* were not retrieved after NaOH treatment, whereas only a few *M. mucogenicum* and almost all *M. chimaeras* were recovered (Figure 1). One can hypothesize that conversely to water without organic materials, the mucus and human cells contained in sputum protect NTM from NaOH action, [8]. Interestingly, a study on the isolation of *Mycobacterium tuberculosis* in human samples showed that combining CPC with NaOH could not only reduce the concentration of NaOH used for

decontamination, but also improve the sensitivity of the analysis without altering its performance [11]. Given that NTM were sensitive to NaOH and that 0.005%-CPC yielded satisfactory results, we decided not to test this combination. Indeed, we were unsure that combining NaOH with CPC would improve the decontamination method. To conclude, in the detection of the seven NTM species studied herein, CPC offers a better survival rate than NaOH, and we recommend its being used to decontaminate water samples.

Detection limit after CPC decontamination and filtration in artificial tap water

Up until now, NTM tolerance to CPC was always tested on NTM suspension in sterile water, but not in real-life conditions [2,12]. Here, the detection limit was determined on a NTM suspension in a tap water-like sample containing a mix of mesophilic bacteria so as to mimic real-life conditions. We cannot directly use tap water insofar as it may already contain NTM [13], which would interfere with the detection of artificially spiked NTM. With that in mind, we tested the 0.005%-CPC conventionally used to decontaminate low-contaminated water [4,14]. As expected, control cultures of artificial tap water samples containing NTM were invaded by mesophilic bacteria after 3 days of incubation, which rendered NTM isolation and identification impossible. However, CPC treatment turned out to be effective as a means of decontaminating interfering mesophilic bacteria. NTM detection limits in artificial tap water combining 0.005%-CPC decontamination and filtration ranged from 3 CFU/100mL for *M. abscessus* to 98 CFU/100mL for *M. wolinskyi* (Table 1), showing that our method was an effective means of detecting NTM in polymicrobial water samples. However, at higher concentration (0.05%), CPC is too aggressive to preserve NTM in water [3,4].

Previous studies testing NTM sensitivity to CPC used different concentrations of the latter, rendering it difficult to compare their respective results [5,9,10]. In this study, at a concentration of 0.005%-CPC, NTM sensitivity varied between species (Table 1). *M. smegmatis* was more susceptible than *M. abscessus* and *M. chelonae*, a finding consistent with a previous study [13]. However, variation in detection limit does not seem to be due exclusively to NTM susceptibility to CPC. For instance, *M.*

chelonae is more accurately detected than *M. llatzerense* despite a lower survival rate in the presence of CPC. This incongruence could be explained by the high propensity of NTM to form aggregates, which could differ according to species and might bias comparison between them. In the future, other NTM species and different strains of a same species should be tested in order to determine whether, as previously shown, CPC tolerance depends on species or strain [5,12].

To conclude, interpretation of the results of water analyses using CPC should consider the detection limit for every NTM, as an absence of cultivable NTM cannot ensure their being totally absent from water. However, our results show that 0.005%-CPC can yield sufficient detection, at least for the seven NTM species we have studied. To our knowledge, a better decontamination process that effectively eliminates mesophilic bacteria while completely preserving all NTM species is not available yet. We therefore propose to search NTM in water by means of decontamination with CPC at 0.005%, filtration concentration, and direct culture of membrane onto Middlebrook 7H10 media at 30°C for at least 2 weeks. Under real-life conditions, this protocol demonstrated its effectiveness during our analyses of water samples from hospital or domestic environments, and it seems especially adapted for environmental investigation of HAIs caused by the two main NTM, *M. chelonae* and *M. chimaera*.

Funding source

None to declare

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Figure 1: Comparison between Non-Tuberculous Mycobacteria survival rates after treatment by cetylpyridinium chloride or sodium hydroxide in water samples

CPC, cetylpyridinium chloride; NaOH, sodium hydroxide; NTM, Non-Tuberculous Mycobacteria; ***, p<0.001; **, p<0.01; *, p<0.05.

Experimentations were repeated three times for CPC and filtration assessment, and in duplicate for NaOH decontamination. Survival rate corresponds to the mean ~~of~~ ratio between the number of CFU recovered with or without decontamination treatment. CFU numeration was assessed at day 14 for *M. chimaera* and day 10 for the other NTM species.

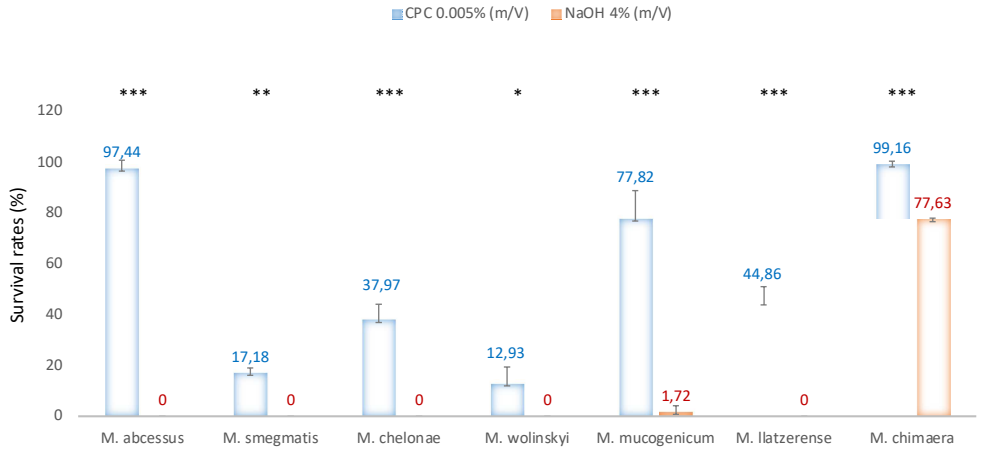

Figure 1: Comparison between Non-Tuberculous Mycobacteria survival rates after a treatment by 0,005% cetylpyridinium chloride or 4% sodium hydroxyde in water samples
CPC, cetylpyridinium chloride; NaOH, sodium hydroxyde; NTM, Non-Tuberculous Mycobacteria; ***, *p<0.001*; **, *p<0.01*; *, *p<0.05*
Experimentations were repeated three times for CPC and filtration assessment, and in duplicate for NaOH decontamination. Survival rate corresponds to the mean of ratio between the number of CFU recovered with and without decontamination treatment. CFU numeration was assessed at day 21 for M. chimaera and day 10 for the other NTM species. Comparison between recovery rates was assessed by Student t-test.

Table 1 : Detection limit of a method combining CPC 0.005% decontamination and concentration by filtration for the detection of seven Non-Tuberculous Mycobacteria species in artificial "tap water" samples

NTM species	Stock NTM suspensions Mycobacterial concentration (CFU/100mL)	culture result of dilution series for 50mL of artificial "tap water" sample			Detection Limit (CFU/100mL)
		10^{-1}	10^{-2}	$2.5\ 10^{-3}$	
Mycobacterium smegmatis	2400	D	D	ND	24
Mycobacterium wolinskyi	980	D	ND	ND	98
Mycobacteria chimaera	1120	D	D	ND	12
Mycobacterium llatzerense	1580	D	D	ND	16
Mycobacterium mucogenicum	980	D	D	ND	10
Mycobacterium abcessus	978	D	D	D	3
Mycobacterium chelonae	610	D	D	ND	7

D, Detected ; ND, Not Detected
NTM Non-Tuberculous Mycobacteria
CFU Colony-Forming Unit
CPC Cetylpyridinium Chloride
Artificial tap water done with filtered tap water spiked with hydric mesophilic bacterial strains (*Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Sphingomonas paucimobilis, Methylobacterium sp., Pseudomonas stutzeri*) at a concentration of 2, 050 CFU/100mL.
50mL of each NTM suspension were decontaminated by a CPC solution (0.005% m/V) for 30 minutes, the mix was filtered onto 0.45µm-porosity cellulose nitrate membrane, which was directly plated onto the growth media (Middlebrook 7H10 agar media plate for *M. chimaera* and Columbia agar media plate for the other NTM species). Medias were incubated at 30°C and CFU detection was assessed at day 21 for *M. chimaera* and day 10 for the other NTM species.
Detection limit : the weakest concentration allowing detection of NTM.
Experimentations were repeated two times for each NTM species.