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Optimized Method for Isolation of Nontuberculous Mycobacteria from Hospital Aquatic Sources

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ABSTRACT

Background: Aquatic ecosystems are an important source of nontuberculous mycobacteria (NTM) that can cause different diseases in human. Since culture of mycobacteria needs long-term incubation, fast-growing microorganisms and contaminants in the environment usually prevents the isolation of mycobacteria. Here, we compare different treatment protocols and describe a method that increases the recovery and improve the culturability of NTM from aqueous samples.

Materials & Methods: A total of 35 samples from the water sources like tap water, and medical devices such as manometer, dialysis devices, nebulizers, ventilator and dental units were collected. Containers containing 50 mL of the sample were immediately transferred for culture on Lowenstein-Jensen medium to the laboratory and examined. For better isolation of NTM, different concentrations of NaOH, sodium dodecyl sulphate (SDS), cetylpyridinium chlorid (CPC), oxalic acid and cyclohexamide in culture media were examined.

Results: Culture media with 1% solution of NaOH, 3% SDS and 5% oxalic acid was completely effective to eliminate the contaminants and it also showed the lowest inhibitory effect on mycobacteria. The concentrations between 0.3 gr to 1 gr of cyclohexamide had the best inhibitory effect on growth of fungi.

Conclusion: Culture media with NaOH 1%, SDS 3%, 5% of oxalic acid and 0.3-1 gr cyclohexamide can increase the recovery and improve the culturability of NTM from aqueous samples.

Keywords: Nontuberculous mycobacteria, Hospital water sources, Isolation protocols

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Introduction

Nontuberculous mycobacteria (NTM) are opportunistic pathogens for humans. More than 160 species of Mycobacterium genus have been recognized and they have high variability in the aspects of virulence, adaptation to the environment, pathogenicity, drug resistance and growth characteristics **(1)**. A variety of NTM often widely dispersed in the environment can cause opportunistic infections in human **(2-4)**. Environmental resources including water, soil, dust and aerosol **(5)** and in many cases, water is a carrier of the bacteria. Most NTM infections can cause disease through contact with soil and water resources and human-to-human transmission is not reported until now [6]. Although the contact between the human and environmental mycobacteria is inevitable, the disease by NTM mostly related with skin defect, having previous

lung disease and disorders such as congenital and acquired immunodeficiency.

These bacteria are mostly resistant to antibiotics, antiseptics and disinfectants and are also considered as nosocomial pathogen **(7)**. Currently, more than 20 NTM species have been identified in drinking water that are resistant to disinfectants (such as chlorine) and also tolerate a wide range of pH and temperature. In addition, NTM can survive in pipes containing flowing water due to biofilm formation, their hydrophobicity and amoeba-associated lifestyle **(5, 8-13)**. The bacteria also were isolated from hot water systems, spas and swimming pools. Since culture of mycobacteria needs long-term incubation, fast-growing microorganisms and contaminants in the environment usually prevents the isolation of mycobacteria. For this reason, different treatment protocols were examined to increase the recovery and improve the culturability of NTM from aqueous samples.

Materials and Methods

Sample Collection

A total of 35 samples from the aquatic sources of the teaching hospitals of Iran University of Medical Sciences, Tehran, Iran, including tap water and medical devices such as manometer, dialysis devices, nebulizers, ventilator and dental units were collected. Approximately 50 mL of each sample was collected in a sterile glass bottle, transferred to laboratory in an icebox and examined within 24 hrs.

Sample Preparation and Culture

Water samples were centrifuged for 30 minutes at 3000 rpm. The sediment (3 mL) was transferred to two sterile containers, and was decontaminated by two methods. The first method was implemented as following: 1.5 mL of 1% NaOH and 1.5 mL of 3% SDS were added for decontamination after centrifuge and were incubated at room temperature for 30 minutes. Then, Phenolphthalein reagent and 40% phosphoric acid were added for neutralization. The tubes were then centrifuged at 3000 rpm. Supernatant was discarded and the pellet was cultured on two separate tubes containing Lowenstein Jensen (LJ) media which were then placed at 25°C and 37°C. Second method was as following: 0.05% CPC solution (cetylpyridinium chloride) with a volume equal to the sample was added after centrifugation. Samples were shaked for 20 minutes at room temperature and then centrifuged at 3000 rpm for 15 minutes and supernatant was discarded. Then, 2 mL of distilled water was added to the pellet for neutralization of the remaining CPC and was further centrifuged at 3000 rpm. Finally, pellet was cultured on two separate tubes of LJ media and placed at 25°C and 37°C. Culture media were studied every 48 h for 5 months for the possible growth of colonies. Then, Acid-fast staining was performed by Ziehl-Neelsen method which confirmed the presence of Mycobacteria. Phenotipycal characteristics and molecular method were used for confirmation **(14)**. For high contamination of samples with yeast and fungi, different concentrations of cyclohexamide were added to LJ media for primary isolation of the bacteria. Different cyclohexamide concentrations were 0.025 g, 0.05 g, 0.06 g, 0.64 g, 0.2 g, 0.3 g, 0.5 g and 1 g, were used for each 1600cc of LJ culture media.

Another treatment protocol was conducted for the isolation of colonies on contaminated LJ culture media with other bacteria or fungi by following four methods: First protocol; Mixed colonies were picked off and dissolved in 200 µL of distilled water in a sterile falcon. Then, CPC with the volume equal to the sample was added and vortexed. It was then shaken at room temperature for 30 minutes and centrifuged at 3000 rpm for 30 minutes. In the next step, supernatant was discarded and 200 µL of distilled water was added to the sample for washing and was again centrifuged at 3000 rpm for 15 minutes. Supernatant was discarded and finally, the pellet was cultured on LJ culture media containing cyclohexamide. Second protocol; Mixed colonies dissolved in 200 µL of distilled water in a sterile falcon and 1.5 mL of 1% NaOH and 1.5 mL of 3% SDS was then added. After vortexing and shaking at room temperature for 30 minutes, phenolphthalein reagent and 40% phosphoric acid were added for neutralization. Sample was then centrifuged at 3000 rpm for 30 minutes. Supernatant was discarded and the pellet was cultured on LJ media containing cyclohexamide. Third protocol; mixed colonies dissolved in 200 µL of distilled water in a sterile falcon. Then, 200 µL of 5% oxalic acid was added to the solution. After vortexing, it was shaken at room temperature for 30 minutes. At that point, sample was centrifuged at 3000 rpm for 30 minutes. Supernatant was discarded and pellet was cultured on LJ media containing cyclohexamide. Fourth protocol; mixed colonies dissolved in 200 µL of distilled water in a sterile falcon. 1.5 mL of 1% NaOH and 1.5 mL of 3% SDS were then added. After vortexing, it was shaken at room temperature for 30 minutes. Phenolphthalein reagent and 40% phosphoric acid were added for neutralization. Sample was then centrifuged at 3000 rpm for 30 minutes. Supernatant was discarded and the pellet was vortexed and 200 µL of 5% oxalic acid was added. The tube was then shaken at room temperature for 40 minutes and finally, sample was centrifuged at 3000 rpm for 30 minutes. Supernatant was discarded and the remaining pellet was cultured on LJ media containing cyclohexamide.

Also, different culture media were used for isolation of Mycobacterium colonies from contaminated LJ media. The media was including: BHI agar culture

media, MacConkey culture media, Blood agar containing nalidixic acid (0.056 gr per 1600 cc of culture media), Blood agar containing penicillin G (0.05 gr), Blood agar containing nalidixic acid and penicillin (0.056 gr and 0.05 gr, respectively), LJ culture media containing nalidixic acid, penicillin and cyclohexamide (0.02 gr per 1600 cc culture media).

Results

Among the used protocols, mycobacteria were isolated by the two methods that are shown in Table 1. Concentrations of 0.025, 0.05 and 0.06 were somewhat effective in reducing fungal and yeast contaminations but did not thoroughly prevent contamination. Concentrations of 0.64 to 0.2 eradicated more than half of the fungal and yeast contaminations and in concentrations of 0.3 g and 0.5 g no fungal contamination was observed. But in some cases, small amounts of yeast and fungal contamination were still seen which were completely removed by concentration of 1 g cyclohexamide.

Treating the contaminated colonies by 1-0.05% CPC solution and 2-1% NaOH and 3% SDS protocols did not reduced the contamination. Threating with 3-5% oxalic acid reduced the contamination but was not completely removed and after several days contamination level raised and other bacterial and yeast agents grew along with Mycobacteria. Finally, 4-1% NaOH, 3% SDS and 5% oxalic acid protocol was completely removed contamination from all the treated media and mycobacteria grew purely.

Table 1. Frequency of mycobacterial isolates in water sources of the hospitals

Sample water	Temperature	Positive	Negative	Contaminated
NAOH+SDS	37°C	19	9	7
	25°C	24	7	4
CPC 0.05	37°C	0	33	2
	25°C	8	26	1

On BHI agar, no growth was observed after two months of storage. Culture on MacConkey agar and blood agar containing nalidixic acid and penicillin. A few days after culture, Gram-negative bacteria had growth on all culture media and no growth of mycobacteria was observed. On selective LJ culture media with antibiotics, no contamination was observed and mycobacteria started growth after a month or even two months in some cases but the rate of Mycobacterial growth was lower compared to LJ media containing cyclohexamide without antibiotics (penicillin and nalidixic acid).

Discussion

Today, many studies emphasize on the necessity of identifying NTM in water and finding the suitable solution for controlling these contaminations **(14)**. There are different methods for isolation of NTM from the environment but there is a need for a standard method in order to identify the biological sources contaminated with NTM. In a study conducted by Kamala *et al.* in 1993 in India, 6 decontamination methods were used for isolation of mycobacteria from water and soil. More positive samples were detected by 3% SLS (Sodium Lauryl Sulfate) and 1% NaOH **(15)**. Also, in a study conducted by Nicolas Radomski in 2009, it was shown that decontamination with 0.05% CPC for 30 minutes and culture on rich LJ media containing PANTA, reduces the growth of unwanted bacteria significantly. It was shown that decontamination with 0.05% CPC caused termination of *Mycobacterium chelonae* (71.1%) and *Mycobacterium avium* (70%). In contrast, in a study conducted by Thomson *et al.* it was shown that using 0.005 CPC in water samples leads to the survival of 3.6% of both *M. avium* and *Mycobacterium intercellular* **(16)**. However, CPC could be used in different concentrations for samples with low (0.005%) or high (0.05%) contaminations **(17)**.

In a study by Khosravi and *et al*. in 2016 in Khuzestan province of Iran, 77 culture positive mycobacteria were isolated from 258 hospital water samples. CPC 0.005% was used for decontamination **(18)**. Moreover, in a study by Falsafi *et al.* in Iran, three methods of decontamination including 0.01% CPC, 4% NaOH and 1% NaOH+3% SDS were used and indicated that decontamination with CPC is the best method for decontamination which reduces the growth of unwanted microorganism and does not have much

inhibitory effects on NTM (19). Rahbar *et al.* in Iran in 2010 were studied on 120 water samples, they were able to isolate 10% of the total samples by 0.05% CPC (20).

According to the results of the current study, the use of lower concentrations of CPC was highly effective compared to 1% NaOH and 3% SDS as using 0.05% CPC resulted in less contamination and higher persistence of samples compared to NaOH and SDS. But the number samples with Mycobacterial growth was lower. Most media with CPC showed no growth which is estimated to be a result from using high concentrations of CPC which probably is more effective in lower concentrations. Meanwhile, the growth rate of mycobacteria increases by increasing cyclohexamide level in culture media and no inhibitory effect was observed in Mycobacterial growth in cyclohexamide concentration to 1 g per 1600 cc. However, concentrations of 0.3 g and 0.5 g were the most effective in the purification of NTM. Nalidixic acid was effective in the preventing the contamination of culture media and purification of mycobacteria but can somewhat prevent the growth of Mycobacteria. Nalidixic acid has the highest inhibitory effect on NTM when the number of mycobacteria is low in the sample. Therefore, it is suggested to use nalidixic acid for purification and not for primary decontamination. In many studies conducted in Iran, 1% NaOH and 3% SDS were used same as the current study. But it seems like using low concentrations of CPC is effective in the isolation of NTM and depend on sample volume.

In conclusion, the use of NaOH 1%, SDS 3%, 5% of oxalic acid and 0.3-1 gr cyclohexamide can increase the recovery and improve the culturability of NTM from aqueous samples.

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Conflict of Interest

Authors declared no conflict of interests.