Efficacy of Calcium Hypochlorite and Ultraviolet Irradiation against *Mycobacterium fortuitum* and *Mycobacterium marinum*

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**Abstract**

**Background:** Nontuberculous mycobacteria (NTM) cause opportunistic infections with increasing frequency in immunocompromised humans. Water is one of the natural sources for transmission of NTM and plays a major role in the epidemiology of NTM infections. This study evaluated the efficacy of calcium hypochlorite and ultraviolet irradiation (UV) to eliminate potentially zoonotic NTM species such as *M. marinum* and *M. fortuitum*. **Materials and Methods:** Bacterial suspensions containing 1-4 × 10⁷ CFU/ml were exposed to 5, 50, 100, 1,000 and 10,000 mg/L of Ca (OCl), for 1, 5, 10, 15, 20, 30 and 60 minutes, and 6,000 µW/cm² UV dose for 5, 10, 20, 30, 60 and 120 seconds. **Results:** Of the two methods tested, UV irradiation was more effective than chlorine in achieving three log reduction in viable bacterial count (UV dose 6,000 µW/cm², exposure time 60 S) as well as in eliminating the organisms (UV dose 17,000 µW/cm², exposure time: 30 S). When 10,000 mg/L of chlorine was used, 10 and 20 min contact times were required to achieve three log inactivation and complete elimination of *M. fortuitum* respectively. **Conclusion:** Our study suggest that initial disinfection of water by chlorine at the water treatment plant followed by UV irradiation at the household level would minimise the spread of NTM to the susceptible population via drinking water. **Keywords:** Chlorine, *Mycobacterium fortuitum*, *Mycobacterium marinum*, nontuberculous mycobacteria, UV irradiation

**Introduction**

Nontuberculous mycobacteria (NTM) are usually present in soil, water, and aerosol. Certain species of NTM could cause opportunistic infections in humans and animals characterized by pulmonary infections and lymphadenitis as well as the disseminated infections in the skin, soft tissues, and bones.¹⁻⁴ Currently, there is an increasing trend in the occurrence of NTM infections in humans, especially those with immunodeficiency disorders.⁵⁻¹⁰ Human-to-human transmission does not usually occur and the infection is thought to be acquired from the environment by ingestion, inhalation, or inoculation.¹¹ Many studies identified that the contaminated water is the main source of NTM infections in humans.³⁻⁵,⁹⁻¹⁵ NTM species are undoubtedly contributing a considerable proportion of pulmonary and extrapulmonary tuberculosis in human in Sri Lanka and appropriate measures need to be taken to minimize the spread of these organisms in the country.²¹⁻²² As there is no effective treatment against NTM infections, appropriate measures need to be taken to minimize the spread of these organisms among humans.

Often, the microorganisms present in drinking water are eliminated by mechanical (filtration, sedimentation, coagulation, or flocculation) and chemical methods. Many of the NTM species are resistant to most of the disinfectants used in water treatment and surface and instrument disinfection,

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perhaps owing to the impermeability of the cell wall or the formation of biofilm. nowadays, ultraviolet (uv) light is being increasingly used to inactivate certain microorganisms as it would not leave any harmful disinfection by-products.

mycobacterium marinum and mycobacterium fortuitum are potential human pathogens representing slow- and fast-growing NTM species, respectively. Isolation of these two species from different water sources has been recorded frequently. The present study determined the efficacy of calcium hypochlorite (Ca(OCl)2) and UV irradiation against these two commonly isolated NTM species.

Materials and Methods
Preparation of the bacterial suspension
The strain of M. fortuitum and M. marinum used in this study has been isolated from water in a previous study. The isolates were separately inoculated in Middlebrook 7H9 Broth containing 10% (v/v) oleic acid albumin enrichment and 0.05% Tween 80 and incubated at 37°C for 7 days, and the bacteria were harvested by centrifugation at 800 × g for 10 min. The bacterial pellets were washed twice and suspended in distilled water to adjust the optical density (OD) to 0.1 at 600 nm wavelength (approximately 1–4 × 10^8 CFU/ml).

Effectiveness of calcium hypochlorite against Mycobacterium marinum and Mycobacterium fortuitum
Effectiveness Ca(OCl), against M. marinum and M. fortuitum was determined according to Mainous and Smith. Briefly, Ca(OCl), solutions were prepared at the concentrations of 0, 5, 50, 100, 1000, and 10,000 mg/L in sterile distilled water immediately before use. A volume of 100 µl of M. fortuitum (0.1 OD at 600 nm wavelength) was added to six tubes each containing 900 µl of Ca(OCl), at different concentrations as mentioned above. Accordingly, seven sets of experimental tubes were prepared and incubated for 1, 5, 10, 15, 20, 30, and 60 min, respectively. After completion of the incubation time, each sample was subjected to 10-fold dilutions in distilled water and 10 µl was immediately plated on Middlebrook 7H10 agar in duplicates. The plates were incubated for a minimum of 7 days at 37°C, and the resulting colonies were counted. The experimental protocol to test the efficacy of Ca(OCl), against M. marinum was essentially the same, except the cultures were incubated at 25°C for 12 days. The above two experiments were repeated again to obtain mean values.

Effectiveness of ultraviolet light treatment against Mycobacterium marinum and Mycobacterium fortuitum
This experiment was performed at room temperature (25°C) and at pH 7.0 according to Hayes et al. A bench-scale collimated beam apparatus was used for UV irradiation. Two 15 W low-pressure UV lamps (Model G15T8, American UV Co., IN, USA) were the light source and a manually operated switch was employed to control the length of UV exposure. The reactor was flat-bottomed glass Petri dish with an inner diameter of 90 mm and a radiometer (UV light meter) (Model ST 512, Sentry Optronics Corp., Taiwan) was used to measure the irradiance (E) in microwatts per square centimeter (µW/cm²).

Exposures spanned a UV dose ranging of approximately 6000–17,000 µW/cm². Five milliliters of M. fortuitum suspensions (1–4 × 10^5 CFU/ml) was transferred to six Petri dishes and irradiated using 6000 µW/cm² UV dose for six different UV light times (5, 10, 20, 30, 60, and 120 s, respectively). After the UV irradiation, the viable CFU was determined as described above. Nonirradiated samples were also plated to determine the initial organism levels (N₀). The resulting colonies were counted and calculated the number of CFU in 1 ml of water. The above experiment was also conducted with two additional UV doses (10,000 and 17,000 µW/cm²) in a similar manner. The experimental protocol to test the susceptibility of M. marinum for three different levels of UV exposure was essentially the same as described above. All of the above experiments had two replicates to obtain mean values.

Results
Inactivation of Mycobacterium fortuitum and Mycobacterium marinum by calcium hypochlorite
M. fortuitum was found to be more resistant to Ca(OCl)₂ than M. marinum. When low (5 and 10 mg/L) and moderately high (100 mg/ml) concentrations of Ca(OCl), were used, only 10-fold reduction in viable M. fortuitum count was achieved after 60 min of exposure time. To achieve three log reduction in viable M. fortuitum count, it was necessary to expose the organism to very high concentration (10,000 mg/L) of Ca(OCl), for 5 min. In contrast, M. marinum showed three log inactivation on 5 min exposure to 5 mg/L Ca(OCl), or 1 min exposure to 10 mg/L concentration. Complete elimination of M. fortuitum was only achieved by exposing the organism to 10,000 mg/L concentration for at least 20 min whereas 5 and 10 mg/L Ca(OCl), completely eliminated M. marinum at the contact time of 20 and 5 min, respectively [Table 1].

Inactivation by ultraviolet irradiation
The time required for three log inactivation of M. fortuitum at the UV doses of 6000 and 10,000 µW/cm² was 60 and 30 s, respectively, whereas for three log inactivation of M. marinum was achieved with the above doses at 10 and 5 s, respectively. Complete elimination of M. fortuitum required longer exposure time (60 or 120 s) than that of M. marinum at the above UV doses. However, at a high UV dose (approximately 17,000 µW/cm²), complete inactivation of M. fortuitum and M. marinum was achieved within 30 and 5 s of exposure time, respectively [Table 2].

Discussion
Mycobacterium species are highly resistant to commonly used disinfectants than any other microorganisms due to thick, hydrophobic cell wall rich in mycolic acids which
Table 1: Bacterial plate counts of Mycobacterium fortuitum and Mycobacterium marinum following exposure to different concentrations of calcium hypochlorite

| Time (min) | Mycobacterium fortuitum | Mycobacterium marinum |
|------------|-------------------------|-----------------------|
|            | Ca(OCl)₂ concentration (mg/L) |                      |                      |
|            | 5                       | 10                    | 100                   | 1000                  | 10,000                 |
| 1          | >1×10⁰                  | >1×10⁰                | >1×10⁰                | >1×10⁰                | 1×10⁴                 |
| 5          | >1×10⁰                  | >1×10⁰                | >1×10⁰                | 1.5×10⁰               | 6.5×10⁴               |
| 10         | >1×10⁰                  | >1×10⁰                | >1×10⁰                | 1×10⁰                 | 5×10⁴                 |
| 20         | >1×10⁰                  | >1×10⁰                | 3×10⁰                 | 8×10⁴                 | NG                    |
| 30         | 3×10⁴                   | 2.5×10⁴               | 1.8×10⁴               | 5×10⁴                 | NG                    |
| 60         | 2×10⁴                   | 1.5×10⁴               | 1×10⁴                 | 3×10⁴                 | NG                    |

Duplicate plate counts were made at each exposure in two replicate trials; the number (CFU/ml) indicates the average bacterial counts. There was no loss of bacterial growth in distilled water controls. Initial bacterial count (CFU/ml)=1×10⁰ (absorbance 600 nm=0.100). NG: No growth, CFUs: Colony forming units, Ca(OCl)₂: Calcium hypochlorite

Table 2: Bacterial plate counts of M. fortuitum and M. marinum following exposure to various doses of UV irradiation

| Time (Sec) | UV dose (µW/cm²) | M. fortuitum | M. marinum |
|------------|------------------|--------------|------------|
|            | 6,000            | 10,000       | 17,000     |
| 5          | 8.7×10¹           | 3.0×10¹      | 2.5×10¹    | 5.0×10⁰               | 7.5×10⁰               | NG                    |
| 10         | 2.0×10¹           | 1.5×10¹      | 1.5×10¹    | 2.5×10¹               | NG                    | NG                    |
| 20         | 1.5×10¹           | 1.5×10¹      | 1.2×10¹    | NG                    | NG                    | NG                    |
| 30         | 7.5×10⁴           | 5.0×10⁴      | NG         | NG                    | NG                    | NG                    |
| 60         | 2.5×10²           | NG           | NG         | NG                    | NG                    | NG                    |
| 120        | NG                | NG           | NG         | NG                    | NG                    | NG                    |

Duplicate plate counts were made at each exposure in two replicate trials; the number of colony forming units per milliliters (CFU/ml) indicates the average bacterial counts. There was no loss of bacterial growth in controls which were not exposed to UV light. Abbreviations are as follows: NG=no growth. Initial bacterial count (CFU/ml)=1×10⁰ (Absorbance 600 nm=0.100)

help them form aggregates in liquid media. A number of studies have shown that the resistance to disinfectants by different species of Mycobacterium varies widely owing to the differences in the chemical composition of cell walls, especially the outer wall, which is species specific. A recent disinfectant study has shown that M. fortuitum is markedly resistant than other NTM to chlorine and survived at 60 min of exposure to 2 µg/mL of free chlorine. Our results are also in agreement with this, and we only achieved 10-fold reduction in viable M. fortuitum count by exposing to 5 µg/mL, Ca(OCl)₂, for 60 min. Complete inactivation of M. fortuitum required concentration of Ca(OCl)₂ as high as 10,000 mg/L (10 µg/ml) and previous studies have also reported the high resistance of M. fortuitum to chlorine. Concentrations of Ca(OCl)₂ which are capable of complete inactivation M. fortuitum are well above the concentrations recommended for drinking water. Thus, these concentrations could only be used to treat waste water contaminated with M. fortuitum. Treating waste water prior to dispose is important to minimize the spread of pathogens into the water environment and drinking water supplies.

Disinfection by chlorination can be problematic, in some circumstances. Chlorine can react with naturally occurring organic compounds found in the water supply to produce disinfectant by-products. There are also other concerns regarding chlorine, including its volatile nature which causes it to disappear too quickly from the water system, and esthetic concerns such as taste and odor. The advantage of chlorine in comparison to other methods of disinfection (e.g., ozonization) is that it offers residual disinfection. This feature allows the chlorine to travel through the water supply system, effectively controlling pathogenic backflow contamination.

Comparatively, UV irradiation was more effective in eliminating Mycobacterium. Complete elimination of M. fortuitum and M. marinum was obtained within 60 s of contact time at a UV dose of approximately 17,000 µW/cm². Lower doses (approximately 10,000 and 6000 µW/cm²) required 120 s of exposure time to achieve complete elimination of M. fortuitum and M. marinum, respectively. A previous study has reported that a UV dose of 66 ml/cm² (66,000 µW/cm²) was essential for three log inactivation of M. fortuitum. Similarly, in the present study, three log reductions of M. fortuitum was obtained at UV dose 6600 µW/cm² with 10 s of exposure time (60 ml/cm²). In a study carried out by Hayes et al., they have obtained four log inactivation of M. avium and Mycobacterium intracellulare at a UV dose <20 ml/cm². UV disinfection of water consists of a purely physical, chemical-free process. UV radiation in particular, with a wavelength in the 240–280 nm range, attacks the vital DNA of the bacteria directly. The radiation initiates a photochemical reaction that destroys the genetic information contained in the DNA. The bacteria lose their reproductive capability and are destroyed. UV water treatment can be applied to aquaria, wells, and surface waters (NDWC, 2000).

UV treatment compares favorably with other water disinfection systems in terms of cost, labor, and the need for technically trained personnel for operation. UV disinfection is quick and clean and leaves no taint. However, the absence of residual disinfection and low penetrability in water containing suspended solids are the major disadvantages of UV irradiation.
in mass scale. A process combining both these methods would bring down the NTM count in drinking water to near zero.

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**Conflicts of interest**

There are no conflicts of interest.

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