Abstract

**Background:** There is evidence that drinking water could be a source of infections with pathogenic nontuberculous mycobacteria (NTM) potentially risky to human health. The aim was to investigate the resistance of two NTM isolated from drinking water, *Mycobacterium gordonae* and *Mycobacterium chubuense*, at different concentrations of chlorine (as sodium hypochlorite), used in drinking water sanitation.

**Methods:** The NTM were grown in suspension and in biofilms and were challenged with biocide for 10 and 60 min. **Results:** To obtain 7-log reduction from the initial population of *M. chubuense*, in the planktonic state, there were necessary 20 ppm of chlorine and 60 min of exposure. The same effect was achieved in *M. gordonae* with 10 ppm for the same period. The maximum reduction of both NTM in biofilm was 3-log reduction and was achieved using 30 ppm for 60 min. The chlorine susceptibility of cells in biofilms was significantly lower than that of planktonic cells. The results highlight the resistance of both NTM to the concentrations used in routine water sanitation (0.2 ppm according to Argentine Food Code). Differences in chlorine resistance found between the two NTM in planktonic growth decrease when they are grown in biofilm. **Conclusion:** This suggests that current water disinfection procedures do not always achieve effective control of NTM in the public supply system, with the consequent health risk to susceptible population, and the need to take into account biofilms, because of their deep consequences in the way to analyze the survival of prokaryotic cells in different environments.

**Keywords:** Biofilm, chlorine resistance, drinking water, nontuberculous mycobacteria, planktonic cells

Introduction

Nontuberculous mycobacteria (NTM), atypical, or simply environmental mycobacteria are emergent pathogens capable of causing a large number of opportunistic infections in humans and animals.[1–3] Epidemiological studies suggest that the main source of contamination in humans is natural and drinking water.[4]

The provision of safe drinking water for the population is a topic of interest and concern in the world. Routine bacteriological analyzes appear to be insufficient to ensure the absence of NTM. These bacteria are normal inhabitants of environment, but they also can be transmitted by inhalation, ingestion, or inoculation from environmental sources[4–7] rather than from person to person.

This becomes an issue to consider due to the increase of immunodeficient people, in the population, product of aging increased life expectancy, immunosuppressive diseases, or medical treatments, being more susceptible to opportunistic infections, such as skin infections, lymphadenitis, and lung disease.[9]

The slow growth of NTM would suggest that they are bad competitors compared to other water microorganisms. However, the high hydrophobicity of their cell surface, composed of a high content of mycolic acids,[9,10] drives the attachment of mycobacterial cells to surfaces or the air–water interface and thus persists in drinking water distribution systems producing biofilms, transforming them into an important proliferation site, and reservoir of opportunistic pathogens.

Because of their complex wall, mycobacterial cells are resistant to a variety of hydrophilic antibiotics and
disinfectants (e.g., chlorine) as well as decontamination methods.\(^1\)

In previous studies in water distribution system of Bahía Blanca city, the presence of NTM has been demonstrated (data not shown) and that some of the species recovered and identified genetically and phenotypically could be potential pathogens for humans.

Among NTM species isolated from the environment, *Mycobacterium gordonae* is commonly recovered from water (concentrations of up to 1000 colony forming units per liter), soil, and unpasteurized milk.\(^{11,12}\) *M. gordonae* has a low virulence compared with other NTM, with a mortality rate of <0.1%, despite its ubiquity in the environment.\(^{13}\) They usually produce infections in children, immunocompromised patients, or patients with underlying diseases. However, *M. gordonae* can also cause disease in immunocompetent patients.\(^{14-16}\)

The presence of NTM in the hospital water supply can be the cause not only of intra-nosocomial infections but also of confusions in the diagnoses, leading to false results, related to the isolation of bacteria from water and outside the clinical sample. The presence of *M. gordonae* in different water sources (groundwater, distilled water, water from ice machines and drinking water) has led to the report of pseudo outbreaks by several authors.\(^{17-22}\)

*Mycobacterium chubuense* was isolated for the first time in the garden soil of Hospital Chubu, Japan.\(^{23}\) and its presence has been reported in surface water\(^{24}\) and drinking water.\(^{25}\)

The aim of the present work was to investigate *in vitro*, the resistance of *M. gordonae* and *M. chubuense*, in planktonic state (free-swimming organisms) and forming biofilm, at different concentrations of sodium hypochlorite, which is the germicide used in our country for drinking water sanitation.

**Methods**

**Mycobacterial strains**

A strain of *M. gordonae* (slow growing and scotochromogenic)\(^{10,20}\) and a strain of *M. chubuense* (rapid growing and scotochromogenic) were tested. Both isolated from the distribution system water of Bahía Blanca city, Argentina.\(^{25}\)

**Preparation of mycobacterial samples used in the biofilm and planktonic assays**

The bacteria were grown in Löwenstein-Jensen medium (Britania, Argentina) for 15 days. Single isolated colonies of each strain were suspended in a sterile phosphate-buffered solution (PBS), were centrifuged, washed with a sterile PBS, and suspended until obtaining a turbidity of 0.5 McFarland standard. To determine the initial count of viable cells, decimal dilutions were inoculated in Petri dishes and the inoculation was done by spread on Middlebrook 7H10 (MB) agar medium (Difco) supplemented with 0.2% glycerol (vol/vol).

Quantification of biofilm formation

Before the study of susceptibility to sodium hypochlorite (NaClO), a screening test was conducted to detect if the two NTM isolated from the environment (*M. gordonae* and *M. chubuense*) had the capacity to form biofilm *in vitro*. This assay was based on determining the ability of the cells to adhere to the wells of 96-well plates using a colorimetric technique.

Bacterial suspensions were prepared as in 2.2. The biofilm formation test was carried out following the protocols of O’Toole *et al.*\(^{27}\) and Johansen *et al.*\(^{28}\) with modifications. Briefly, 50 µl of the mycobacterial suspensions was inoculated into 96-well sterile microwell dishes (U-bottom with low-evaporation lid, Becton Dickinson). They were incubated at room temperature for 30 min. Then, 150 µl of sterile tap water was added. Wells as negative controls without bacterial suspension were used. The plates were capped and incubated without agitation in a sealed container with 20 ml sterile distilled water, to prevent drying. They were incubated at 30°C for 10 for *M. chubuense* and 20 days for *M. gordonae* for their growth rate. After incubation, the wells were washed with 250 µl of sterile tap water once (to remove the unattached bacteria) and 200 µl of a 1% crystal violet solution was added (this dye stains the cells but not the dishes). The plates were incubated at room temperature for 30 min, rinsed four times thoroughly and vigorously with water, blotted on paper towels, and scored for biofilm formation. The dye incorporated into the biofilm was suspended and solubilized in 200 µl of ethanol: acetone (70:30). To quantify the biofilm, a microplate reader (Synergy HT) was used. The reading was made at 595 nm. Results were presented as the mean value of the triplicates, subtracting the mean value for the negative control plus standard deviation (SD).

Preparation of disinfectant and neutralizing solutions

From a stock solution of 55 g/L of NaClO, the following working solutions were prepared: 0.5, 1, 2, 3, 4, 5, 8, 10, 12, 14, 15, 20, 25, and 30 ppm (parts per million) for the resistance tests of *M. gordonae* and *M. chubuense* in planktonic state and forming biofilm.

Sodium thiosulfate (Na\(_2\)S\(_2\)O\(_3\)) was used to neutralize the residual effect of free chlorine.

Taking into account the reactions involved, the volume and necessary concentrations of the neutralizer for this purpose were calculated, using the 4:1 ratio (NaClO: Na\(_2\)S\(_2\)O\(_3\)).

Test of toxicity and effectiveness of the neutralizer

To know if the neutralizing was toxic for NTM, a volume of the mycobacterial suspension was incubated at room temperature with nine volumes of Na\(_2\)S\(_2\)O\(_3\) for 5 min. For the quantification, decimal dilutions were spread on MB medium.

To demonstrate the correct neutralization of the disinfectant, 4 volumes of NaClO were mixed with 1 volume of Na\(_2\)S\(_2\)O\(_3\) (for each of the NaClO concentrations tested). 500 µl of the mycobacterial suspension was added. It was incubated for 30 min at room temperature and quantification was performed.
in MB. In both experiences, the obtained values, expressed as colony-forming units per milliliter, were compared with the initial count.

**Evaluation of the effect of sodium hypochlorite on nontuberculous mycobacteria grown in planktonic state**  
Suspensions of each NTM (prepared as in 2.2) were inoculated in tubes with different concentrations of the germicide solution (2.3), giving a 1/10 dilution. The exposure times were 10 and 60 min. Subsequently, it was neutralized with sodium thiosulfate (2.3). Dilutions were cultured in MB, to quantify the surviving microorganisms. The plates were incubated for 30 days at 30°C in a sealed container with 20 ml sterile distilled water, to prevent drying. In all the experiments, a tube without NaClO was included to obtain the initial count. Three independent tests were performed.

**Evaluation of the effect of sodium hypochlorite on nontuberculous mycobacteria grown in biofilm state**  
To allow the development of the biofilm, the suspensions were inoculated in sterile multiwell, polystyrene plates’ flat bottom with low evaporation lid (Falcon) containing sterile tap water obtained from the distribution network of the city. They were incubated for different time periods at 30°C: 21 days for the slow-growing mycobacterium and 12 days for the fast-growing mycobacterium. A plate was used for each strain and for each exposure time (10 and 60 min).

To recover the biofilm formed on polystyrene surface, the liquid content of each well was removed and each well was washed once with sterile water to remove the nonadherent bacteria. Then, on the surface of the biofilm, the different concentrations of NaClO were added. Finally, it was neutralized with the sodium thiosulfate solution. The supernatant suspension was removed from each well, and the biofilm was collected with a sterile Teflon scrapper and suspended in 1 ml of sterile distilled water.

To determine the biocidal action of chlorine, surviving NTM were quantified in MB medium. A control was made for each multiwell plate to obtain the bacterial count in the biofilm without treatment. Three independent tests were performed.

**Statistical analysis**  
Two-way ANOVA with nested factors was used.[29] The main factor was the concentration of the disinfectant and the secondary factor was the contact time. The tests for each strain were made separately, and for this reason, the strains were not included as a factor in the statistical analysis.

One-tailed Dunnett test was used to determine the minimal concentration that make a significant reduction in the bacterial count.[29] This test compares the effect of the different concentrations of the disinfectant with the control (initial count without treatment).

Finally, it was studied whether there was a linear adjustment in any concentration range. In this case, the slope of straight line that relates the log10 of the bacteria count to the concentration of NaClO was calculated.

Due to the experimental design used in the biofilm experiments, a three-way ANOVA with nested factors was used. The factors have different hierarchy: strains, times, and concentrations of NaClO. Like the study in planktonic state, a one-tailed Dunnett test was used and it was verified if there was a linear adjustment. In that case, the slope of the straight line was calculated.

InfoStat version 2017. Grupo InfoStat, FCA, National University of Córdoba, Argentina. P was used on data analysis.

**Results and Discussion**  
The two NTM tested formed biofilm in vitro. Results were expressed as mean OD595 after crystal violet staining of biofilm, plus SD of the mean. The values obtained were: 0.240 ± 0.11 for M. gordonae and 0.391 ± 0.11 for M. chubuense.

The tests demonstrated the correct neutralization of the germicide, and there was no evidence of Na2S2O3 toxicity. Therefore, it was considered that the reduction in the colonies counts was due to chlorine action.

Figure 1 shows the effect of different concentrations of NaClO on M. gordonae and M. chubuense in planktonic growth after 10 and 60 min of contact. The results are expressed as geometric mean of three independent experiments.

**Mycobacterium gordonae**  
The two-way ANOVA showed that there was an interaction effect between the different NaClO concentrations used and the contact times (P = 0.0057, F = 3.97). The analysis was continued using the averages of the disinfectant concentrations for each contact time separately.

The lower concentrations of NaClO (0.5 and 1 ppm) did not significantly reduction independently of the contact time. From 2 ppm, highly significant decreases were obtained with 60 min of contact (P < 0.01 by Dunnett test). To achieve the same effect with 10 min, 3 ppm was needed.

In the concentration range of 0.5–5 ppm, a good linear adjustment was obtained for 60 min (P = 0.8633). The slope
of straight line ($b = -1.4$) that relates the log$_{10}$ of the bacterial count to the NaClO concentrations was determined. This involves that the bacterial count decreases by more than one order of magnitude for each ppm disinfectant increase. When the contact time was 10 min, the linear adjustment for the same range of concentrations was not good ($P = 0.0056$); however, it is clear the decrease in the counts due to the action of the increase in the disinfectant concentration [Figure 1]. From 12 ppm, no bacterial counts were obtained with the method used.

*Mycobacterium chubuense*

The two-way ANOVA did not find evidence of interaction between NaClO concentrations and the exposure times used. The general behavior was similar in both exposure times ($P = 0.7803$, $F = 0.64$).

At the lowest concentrations (0.5–5 ppm), there was no significant effect on the decrease in bacterial counts after 10 min. For that time, a significant reduction was achieved from 8 ppm ($P < 0.05$ by Dunnett test).

When the contact time was 60 min, the reduction of the counts was highly significant from 5 ppm ($P < 0.01$ by Dunnett test). No linear adjustment was found in any range of germicidal concentrations.

As shown in Figure 1, to obtain a decrease of 7 orders of magnitude of the initial population of *M. chubuense*, 20 ppm of NaClO and 60 min of contact were necessary, while the same effect was achieved in *M. gordonae* with 10 ppm for the same time.

Although the results show the greatest resistance of *M. chubuense*, both NTM survived at higher NaClO concentrations than those required by the Argentine Food Code for drinking water (0.2 ppm).

Figure 2 shows the action of NaClO on the biofilms formed by *M. gordonae* and *M. chubuense* in the contact times studied.

The results obtained from the three-way ANOVA did not allow discarding the second order interaction: Strain × time × chlorine ($P = 0.07$). The analysis was continued using the averages of the disinfectant concentrations for each strain and contact time separately.

The effect of NaClO on the counts of *M. gordonae* growing in biofilm was significant just from 10 ppm, for both contact times ($P < 0.01$ by Dunnett test). Something similar occurred with *M. chubuense* since a highly significant effect was obtained from 10 ppm but only when the exposure time was 60 min ($P < 0.01$ by Dunnett test).

The maximum reduction of both NTM in biofilm was around 3 orders of magnitude [Figure 2] and it was achieved with 30 ppm (the highest used in the assay) for 60 min. This result shows that the resistance differences found between the two NTM in planktonic growth decrease when they are in biofilm. This may be due to the inability of the disinfectant to penetrate the matrix formed by the NTM, rather than the intrinsic resistance of each strain.

A linear adjustment was obtained for both NTM in 60 min, between 5 and 30 ppm ($P = 0.4$). The slopes of straight lines ($b$) that relate the log$_{10}$ of the bacteria count to the NaClO concentration were: $B = -0.12$ and $b = -0.10$, for *M. gordonae* and *M. chubuense*, respectively. These results supporting that both NTM behave similar to chlorine when they grow in biofilm.

The slopes express a decrease of 0.1 orders of magnitude of survivors NTM for each ppm increase in the disinfectant.

If the results obtained in the planktonic and biofilm state are compared, it is evident that a concentration of 10 ppm during 60 min is necessary to achieve a significant reduction of the counts of both NTM in biofilm. However, in planktonic state, the same effect is achieved with 2 ppm for *M. gordonae* and 5 ppm for *M. chubuense* in the same time [Figures 1 and 2].

**Conclusion**

Through *in vitro* experiments, the survival of two NTM frequently isolated from the water network of Bahía Blanca city (Argentina), at the high values of NaClO tested, both in the planktonic and biofilm state, was demonstrated.

A decrease in susceptibility was expected from NTM to NaClO when they grew in biofilm, but are highlighted the concentration of NaClO and the exposure time required to achieve a decrease in viability (30 ppm for 60 minutes).

The results were consistent with those found by Steed and Falkinham in a study carried out with *Mycobacterium avium* and *Mycobacterium intracellulare* and with the data obtained of *Mycobacterium phlei* by Bardouniotis *et al.* This resistance could be due to the fact that the cell layers and the extracellular material, characteristic of a biofilm, limit the diffusion and penetration of chlorine. Likewise has been suggested that mycobacteria may vary the fatty acid composition of their cell surface, whether they are grow in suspension or adhere to a surface. This variation in the structure of mycolic acids alters the fluidity of the mycobacterial membrane, and this change could affect the permeability to NaClO. The use of NaClO...
as a disinfectant in water distribution systems could select and lead to the dominance of NTM over other more sensitive microorganisms in these habitats.

It should be mentioned that the Argentine Food Code in its article 982 (Joint Resolution Secretary of Policies, Regulation and Health Relations and Secretary of Agriculture, Livestock, Fisheries and Food No 68/2007 and No 196/2007) establishes a minimum residual chlorine of 0.2 ppm for drinking water. In previous studies, it was determined that the concentration in the network of Bahía Blanca city reached values between 0.2 and 2 ppm, depending on the environmental temperature and the distance to the water treatment plant, the only site of chlorination of the local network. This suggests that current water disinfection procedures do not always achieve effective control of NTM population.

It is considered that the continuous improvement of the quality of drinking water constitutes one of the main preventive actions available in public health. In this sense, it is expected that the present work contributes to the knowledge of potentially risky microorganisms. Although two nonpathogenic NTM were used: a rapidly growing one and a slowly growing one, their behavior in regard to the disinfectant could be useful as a model of other NTM present in the water.

On the other hand, it is important to highlight the need to consider biofilms in future studies because this unique bacterial behavior could have great consequences in the way of analyzing the survival of prokaryotic cells in drinking water distribution systems.

In agreement with Hamilton et al.,[32] it is proposed that in the future, efforts to reduce waterborne diseases include opportunistic pathogens such as Mycobacterium spp., which have the ability to colonize the internal surfaces of pipelines and water storage tanks and are increasingly involved in opportunistic infections.

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Conflicts of interest
There are no conflicts of interest.

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