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Short communication

Bacteriological quality of bottled water obtained from Mexico City small water purification plants: Incidence and identification of potentially pathogenic nontuberculous mycobacteria species

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ABSTRACT

The aim of this study was to determine the bacteriological quality of bottled water samples obtained from small purification plants located in Mexico City and to identify potentially pathogenic nontuberculous mycobacteria (NTM) species found in these samples. All 111 samples analyzed were positive for aerobic mesophilic bacteria (AMB) and 46 (41.4%) did not comply with Mexico's Official Guidelines. Sixty-nine (62.1%) and 23 (20.7%) water samples were positive for total coliforms (TC) and fecal coliforms (FC), respectively. A total of 81 (72.9%) of the water samples exceeded the maximum allowed limit stipulated in the guideline. Thirty-three (29.7%) of the purified water samples were positive for NTM, being recovered a total of 40 isolates. These NTM isolates were identified using three molecular markers (*hsp*65, *rrs* and *rpo*B genes) which corresponded to the fast-growing mycobacteria *M. chelonae* (n = 12), *M. porcinum* (n = 8), *M. senegalense* (n = 5), *M. abscessus* (n = 4), *M. septicum* (n = 4), *M. wolinskyi* (n = 3), *M. mucogenicum* (n = 2), *M. fortuitum* (n = 1) and *M.* sp. (n = 1). In seven purified water samples, two different NTM species were isolated simultaneously. Overall, these results showed that most of the purified bottled water samples analyzed in this study had unsatisfactory microbiological quality and some harbored NTM associated with illness. Our data could hasten health authorities to intensify efforts in the routine monitoring of activities in the purified bottled water industry in order to supply safe and healthy water to the public.

1. Introduction

Since the September 1985 earthquake, the population of Mexico has increasingly consumed bottled water because at that moment, it was not safe to consume water directly from the distribution system (PAHO, 1985). Moreover, the seventh cholera pandemic that occurred during the 1990s also exerted an influence on the high consumption of bottled water in Mexico. This pandemic resulted in 45,977 cases of cholera between 1991 and 2002, with a fatality rate of 1.2% (Sepulveda et al., 2006).

In Mexico, consumption of purified bottled water continues to increase steadily because of public concerns about the possibility of finding microbial and chemical contaminants in tap water. The percentage of households that buy bottled water increased by five percentage points in recent years, moving up from 70.8% in 2015 to 76.3% in 2017 (INEGI Mexico, 2018). Because of this demand, the water bottling industry has been booming throughout the country. In Mexico City alone, the fifth most populated city of the world (World Population Review, 2019a), the number of small water purification plants that sell purified bottled water in 20 L jugs increased by 85%, from 1232 in 2012

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to 2282 in 2018 (INEGI Mexico, 2018). This presentation of bottled water (20 L), is the format of greatest purchase by Mexican households, and 98% of the volume of water is acquired in this way (kantar-worldpanel, 2018).

It is a common belief among people that bottled water is free from impurities, such as inorganic ions, heavy metals, organic compounds and bacteria, which they consider safer than tap water (Abd El-Salam et al., 2008). However, several studies have documented the detection of aerobic mesophilic bacteria and coliforms in bottled water in counts which exceed the national and international limits allowed for potable human consumption water (Pant et al., 2016). There are additional important reports of potential pathogens, such as other enteric bacteria (Pant et al., 2016), being detected in bottled water. The fact that bottled water has been implicated in outbreaks of illnesses (Benedict et al., 2017), also emphasizes the need to ensure good microbial quality for this product.

Nontuberculous mycobacteria (NTM) are environmental microorganisms that can affect human health (Piersimoni and Scarparo, 2009). NTM are classified into two groups based on growth rates: slow- and rapid-growing species. The first group takes > 7 days to culture (e.g., *M. avium* complex and *M. kansasii*), while rapid growers take < 7 days (e.g., *M. abscessus*) (Bonaiti et al., 2015). The incidence of NTM disease is increasing worldwide (Kendall and Winthrop, 2013), in both immunocompetent and immunocompromised individuals (Piersimoni and Scarparo, 2009). Some NTM species can cause pulmonary disease, affect the skin, lymph nodes, the gastrointestinal tract, and can produce disseminated disease in severely immunocompromised individuals (Griffith et al., 2007). It has been reported that water is one of the most important reservoirs of NTM (Maleki et al., 2017). Hence, people are exposed to these mycobacteria when drinking, bathing, and showering (inhalation of aerosols). Some studies have also shown that potable water was the source of infection in patients with NTM disease (Falkinham 3rd, 2011). Nevertheless, to the best of our knowledge, there are no published studies that report the microbiological quality and in particular the presence of NTM in bottled purified water in 20 L jugs produced in small purification plants.

We therefore conducted this study to determine the microbiological quality and the presence and identification of NTM in bottled water samples obtained from small purification plants located in various municipalities of Mexico City, in order to establish whether this kind of drinking water might be presenting a potential health risk to consumers.

2. Material and methods

2.1. Area of study and water collection

The selected area of study was Mexico City, a large urban area of 1525 square km that has almost 8.9 million registered inhabitants. During working hours, this city reaches a population of nearly 21.2 million (World Population Review, 2019b). From August 2016 to June 2017, a total of 111 purified water samples sold in polycarbonate 20 L bottles were purchased from 111 small water purification plants established throughout the municipalities that make up Mexico City. The definition of purified water is "potable water that has been subjected to physical or chemical treatments, that is free of infectious agents, and whose ingestion does not cause harmful effects on health" (NOM-041-SSA1-1993). There are a number of water purification methods in Mexico. Generally, the processes used by water purification plants involve: coagulation and flocculation followed by sedimentation, filtration, chlorination, reverse osmosis, ultraviolet radiation and ozonation (NOM-127-SSA1-1994).

2.2. Chemical and microbiological analysis

Each 20-L bottle containing the purified water was vigorously shaken and the jug mouth was disinfected with 70% ethanol solution before taking the corresponding sample. The pH and chlorine residual concentrations of all water samples were determined by using pH test strips and the N,N-diethyl-*p*-phenylenediamine method (NOM-201-SSA1-2015), respectively. Each sample was tested for the presence of aerobic-mesophilic bacteria (AMB), total coliforms (TC) and fecal coliforms (FC) following the methods approved by the Mexican official guidelines NOM-201-SSA1-2015, and NOM-210-SSA1-2014. All data obtained in this work were analyzed according to the guideline NOM-041-SSA1-1993 (guideline 041), which establishes that purified water must have a pH between 6.5 and 8.5, a free residual chlorine concentration up to 0.1 ppm, does not contain a higher amount of 2 $\log_{10}$ CFU per mL (100 CFU/mL) of AMB and the presence of total coliforms must not be detectable in any 100 mL (< 1.1 MPN/100 mL) of sample.

2.3. Isolation and identification of mycobacteria

Five hundred milliliters of water were filtered using the CORNING® sterile filtration system, which has a membrane of 0.22 μm. Subsequently, the membrane was placed onto Middlebrook 7H10 agar plates (Difco, Becton Dickinson) supplemented with albumin dextrose catalase (Becton Dickinson BBL™), cycloheximide (500 μg/mL) and the PANTA cocktail (Becton Dickinson BBL™) (40 U/mL polymyxin B, 4 μg/mL amphotericin B, 16 μg/mL nalidixic acid, 4 μg/mL trimethoprim, and 4 μg/mL azlocillin). Plates were incubated at 35 °C and were examined daily for the first eight days and thereafter once a week for two months. Once the bacterial growth had been observed on the Middlebrook 7H10 agar, the number of colony forming units (CFU/500 mL) was directly determined in each plate by triplicate and the identification of acid-fast bacilli was carried out by Ziehl-Neelsen stain. Acid-fast bacilli were subcultured on Middlebrock 7H10 agar, labeled by sampling location and with a consecutive number.

Isolates belonging to the genus *Mycobacterium* and to the *M. tuberculosis* complex were identified by two PCR assays previously described (Cobos-Marin et al., 2003). In these PCRs, RAC1 and RAC8, and MTB-F and MTB-R primers were used for identification of the *Mycobacterium* genus and for the isolates belonging to the *M. tuberculosis* complex, respectively. As shown by Perez-Martinez et al. (2008), the amplicon size varies depending of the mycobacteria species, ranging from 934 to 1300 bp. Therefore, by exclusion, mycobacteria isolates that did not belong to the *M. tuberculosis* complex were considered to be NTM. These NTM species were identified by three methods: (i) PCR restriction enzyme pattern analysis (PRA) of the 65-kDa heat shock protein gene (*hsp*65), as described by Telenti et al. (1993); (ii) sequencing of the hypervariable region 2 (V2) of the 16S rRNA gene (Kirschner et al., 1993); and (iii) sequencing of a fragment (723-bp) of the *rpo*B gene (Adekambi et al., 2003). Mycobacterial PRA was performed by PCR amplification of a 439-bp fragment of the *hsp*65 gene by using primers Tb11 and Tb12 (Telenti et al., 1993). PRA results were interpreted with the algorithm described by Telenti et al. (1993), which is available on the PRA Database.

Identification of the mycobacterial species was also carried out by automatized sequence of the hypervariable region 2 (V2) of the 16S rRNA gene and of the 723-bp fragment from the *rpo*B gene. The amplification of the 16S rRNA gene was performed using the RAC1 and RAC8 primers (Cobos-Marin et al., 2003). For the amplification of the *rpo*B gene, the Myco-F and Myco-R primers were used to obtain a product of 723-bp (Adekambi et al., 2003). Both products of PCR were sequenced using the RAC8 (Cobos-Marin et al., 2003) and Myco-F (Adekambi et al., 2003) primers, respectively, and the big dye terminator ready reaction kit (Perkin-Elmer, Inc., Wellesley, MA). The sequences were analyzed by ABI PRISM 310 genetic analyzer system (Perkin-Elmer). Nucleotide sequences were compared to known sequences in the GenBank database by using the Blastn algorithm. Species identifications were based on the 100% similarity cut-off for the 16S rRNA gene and ≥97% for the *rpo*B gene.

Table 1
Quantity and frequencies of aerobic-mesophilic bacteria (AMB), total coliforms (TC) and fecal coliforms (FC) in purified water bottled samples[a].

Microorganisms group	Minimum	Median	Maximum	Frequency (%)	Number of samples outside of the 041 guideline[b] (%)[c]
AMB	1.04	1.6	3.6	111 (100)	46 (41.4)
TC	< 1.1	1.1	> 8	69 (62.2)	69 (62.2)
FC	< 1.1	< 1.1	> 8	23 (20.7)	NA

[a] n = 111. Minimum, median and maximum values are in $\log_{10}$ CFU per mL for AMB and in most probable number (MPN) per 100 mL for TC and FC. NA = not applicable (there is no official guideline for this).
[b] Guideline that establishes that purified water should contain up to 2 $\log_{10}$ CFU per mL (100 CFU/mL) of AMB and the presence of TC must not be detectable in any 100 mL (< 1.1 MPN/100 mL).
[c] Regardless of whether the indicator microorganism was found alone or mixed with other microorganisms.

2.4. Statistical analyses

The phi correlation coefficient (r_{phi}) value was calculated to quantify the relationship between all nominal variables (presence of NTM and AMB, TC, and FC). A p value < 0.05 was considered significant. All statistical analysis was run with the Statistical program SPSS for Windows version 21 (IBM, Armonk, NY).

3. Results

3.1. Chemical and microbiological quality of purified water

Water samples had a pH range of 6.9 to 7.9 and a chlorine concentration of < 0.1 ppm. All samples were thus found to be within the chemical standards range recommended by Mexico's Official Guidelines for purified bottled water. Regarding the microbiological quality, all 111 samples analyzed were positive for AMB (Table 1). Concentrations of AMB ranged from 1.04 to 3.6 $\log_{10}$ CFU/mL, and 46 (41.4%) samples exceeded the maximum allowed limit of 2 $\log_{10}$ CFU per mL (100 CFU/mL) stipulated in the 041 guideline. A total of 69 (62.1%) and 23 (20.7%) water samples were positive for TC and FC, respectively. TC and FC presented limits ranging from < 1.1 to > 8 MPN/100 mL (Table 1). A total of 81 (72.9%) of the water samples (containing only AMB, only TC and both AMB + TC) were outside Mexico's Official Guidelines (Table 2).

3.2. Mycobacteria isolation and identification

NTM were isolated from 33 (29.7%) of 111 water samples, recovering a total of 40 isolates. These positive samples were purchased throughout Mexico City's 12 municipalities (Fig. 1). Concentrations of mycobacteria ranged from 1 to 14 CFU/500 mL. All of the 40 NTM isolates recovered in this study belonged to the rapid-growing *Mycobacterium* group. According to three molecular methods (PRA of the *hsp*65 gene and the sequencing of the 16S rRNA and *rpo*B genes), those NTM isolates corresponded to *M. chelonae* (n = 12), *M. porcinum* (n = 8), *M. senegalense* (n = 5), *M. abscessus* (n = 4), *M. septicum* (n = 4), *M. wolinskyi* (n = 3), *M. mucogenicum* (n = 2), *M. fortuitum* (n = 1) and *M.* sp. (n = 1) (Table 3). Additionally, in each of the seven samples we found two different mycobacterial species at the same time, i.e. two species per sample. The isolated species were *M. chelonae* and *M. fortuitum*, *M. chelonae* and *M.* sp., *M. chelonae* and *M. wolinskyi*, *M. senegalense* and *M. abscessus*, *M. abscessus and M. chelonae*, *M. chelonae* and *M. septicum* and *M. porcinum and M. septicum.*

No correlation between the presence of NTM and the presence of TC ($r_{phi} = 0.101$, $p = 0.287$) was found. However, a significant correlation between the presence of NTM and the presence of AMB ($r_{phi} = 0.213$, $p = 0.025$) and FC ($r_{phi} = 0.207, p = 0.029$) was found.

Table 2
Microbiological indicators for which purified water bottled samples[a] were outside of the 041 guideline.

Microorganisms group	Number of samples outside of the 041 guideline[b] (%)
Only AMB	12 (10.8)
Only TC	35 (31.5)
Both AMB + TC	34 (30.6)
Total	81 (72.9)

[a] n = 111. Aerobic-mesophilic bacteria (AMB), total coliforms (TC).
[b] Guideline that establishes that purified water bottled should contain up to up to 2 $\log_{10}$ CFU per mL (100 CFU/mL) of AMB and the presence of TC must not be detectable in any 100 mL (< 1.1 MPN/100 mL).

4. Discussion

There is a limit to the number and types of organisms permissible in purified bottled water. The 041 Mexican guideline stipulates that aerobic-mesophilic bacteria (AMB) should not exceed 2 $\log_{10}$ CFU per mL (100 CFU/mL) and that there should be no coliforms present per 100 mL of water. In our study, the bacteriological quality of purified bottled water obtained from small water purification plants was evaluated, and 72.9% of purified bottled water samples did not comply with the 041 guideline. These results are similar to those published by Pant et al. (2016) who found that 87.5% of bottled water samples in Dharan, Nepal did not follow the WHO microbiological criteria for drinking water (< 100 CFU/mL of AMB and absence of coliformes). In contrast, our results were higher than those found by Abd El-Salam et al. (2008) and Halage et al. (2015) who reported that 54.8% of bottled water samples evaluated in Egypt and only 15% of bottled water samples from Kampala, Uganda, respectively, exceeded the WHO guidelines.

As mentioned before, all water samples contained AMB, and 46 (41.4%) of them were outside of the 041 guideline. Other developing countries such as Egypt and Nigeria reported similar results (Abd El-Salam et al., 2008). Nevertheless, the economic scenario of a particular country appears not to be the reason for not controlling the microbiological quality of purified water, because only 10% of bottled water samples analyzed in Ghana presented AMB levels higher than those recommended by the WHO guideline (Osei et al., 2013). It is also important to note that the presence of aerobic mesophilic bacteria (AMB) in the water in such large numbers (as found in our study), even though not pathogenic, is nevertheless of significance for immune-compromised persons in whom they may cause severe infections.

Other groups of microorganisms which should be determined in order to comply with the official guideline for water quality are the total coliforms (TC) and/or the fecal coliforms (FC). Once one of the groups is detected (regardless of their numbers) it means that human fecal contamination with disease causing agents has occurred (WHO, 2017). In our study, a total of 62.1% of water samples were positive for TC. Our results are higher than the results reported in Egypt, Nigeria and Nepal (from 5.2% - 28.6% of samples) (Abd El-Salam et al., 2008; Pant et al., 2016). In Chennai, India, a study carried out by Venkatesan et al. (2014) reported that none of the bottled water samples analyzed contained TC.

Regarding FC, we found 20.7% of positive samples. In contrast, no purified water samples were found to be positive for FC in Egypt, Uganda, and Nepal, Ethiopia and India (Abd El-Salam et al., 2008;

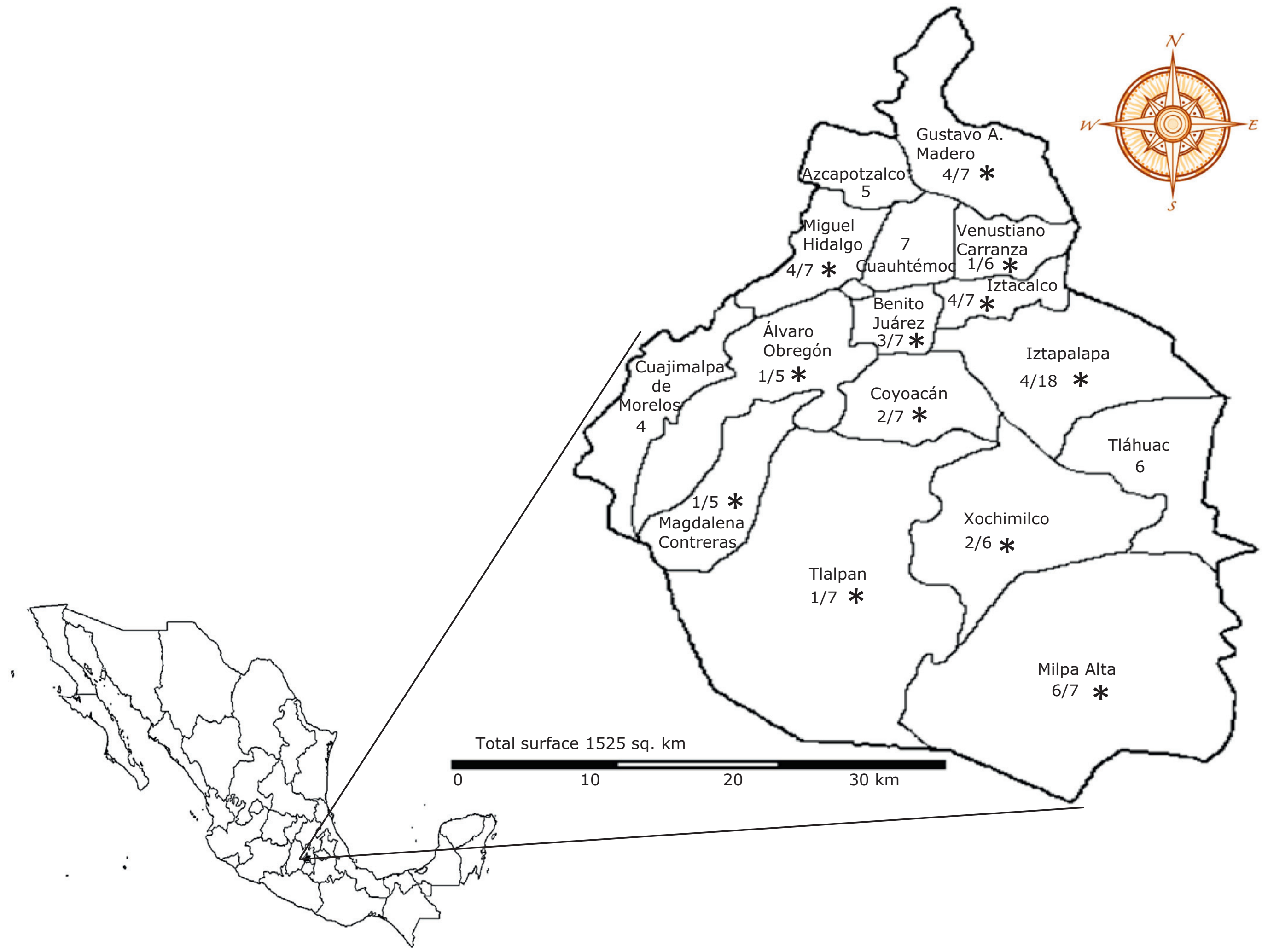

Fig. 1. Mexico City administrative division. Municipalities where the purified water samples contained NTM (*). The quotient represents the number of water samples positive for NTM divided by the total number of water samples collected. Numbers represent the number of samples collected in the corresponding municipality.

Table 3
Nontuberculous mycobacterial species identified in the purified water samples purchased in various municipalities of Mexico City.

Identified species of NTM	No. of isolates (%)	Municipalities where NTM were isolated (%)
M. chelonae[1]	12 (30.0)	8 (50.0)
M. porcinum[2]	8 (20.0)	6 (37.5)
M. senegalense[3]	5 (12.5)	3 (18.8)
M. abscessus[4]	4 (10.0)	4 (25.0)
M. septicum[5]	4 (10.0)	2 (12.5)
M. wolinskyi[6]	3 (7.5)	2 (12.5)
M. mucogenicum[7]	2 (5.0)	2 (12.5)
M. fortuitum[8]	1 (2.5)	1 (6.3)
M. sp.	1 (2.5)	1 (6.3)

From February 2018, there is a new suggested nomenclature for NTM species as follows: [1]*Mycobacteroides chelonae*, [2]*Mycolicibacterium porcinum*, [3]*Mycolicibacterium senegalense,* [4]*Mycobacteroides abscessus,* [5]*Mycolicibacterium septicum,* [6]*Mycolicibacterium wolinskyi*, [7]*Mycolicibacterium mucogenicum* and [8]*Mycolicibacterium fortuitum* (Gupta et al., 2018).

Halage et al., 2015; Pant et al., 2016). Considering the high number of samples identified as outside of the guidelines, we suggest that further studies to monitor the quality of water before and after the purification process should be carried out by the Mexican health authorities. We also recommend that water plant workers be checked continuously regarding their habits of hygiene, because they might be carriers of disease causing-enterobacteria. In parallel, the lack of stringent treatment of containers prior to their being filled with the purified water might represent another possible reason (Oliphant et al., 2002) to explain the high number of AMB and FC which we found in our study. Mexico's Official Guideline (NOM-041-SSA1-1993) stipulates that "the washing and disinfection of 20L containers must be done with sanitizing solution" but it does not specify the required type of sanitizer or at what concentration these agents should be used. In our study, we do not really know which specific sanitizers these Mexican small water purification plants used, if any. If they did use sanitizer, we have no way of knowing which concentration was used. For this reason, we propose that the Mexican Health Authority should clearly establish in their official guideline which types of sanitizers and their respective concentrations should be used for washing the containers used for bottling purified water in order to prevent further contamination.

Recently, Gupta et al. (2018) reported comprehensive phylogenomics and comparative analyses of 150 genomes of mycobacteria species, in order to understand their interrelationships. The compelling evidence from Gupta's study has formed the basis for the division of the genus *Mycobacterium* into five genera. In this classification, the genus name *Mycobacterium* is retained for the clade containing all major human and animal pathogens (e.g., ***M. tuberculosis***, ***M. leprae***, ***M. bovis***, etc.), whereas species from other clades, harboring mainly non-pathogenic species, are transferred into four new genera: *Mycolicibacterium*, *Mycolicibacter*, *Mycolicibacillus*, and *Mycobacteroides*.

Although we isolated NTM in 29.7% of the water samples we tested, this percentage may represent an underestimation of the amount of NTM found in these samples, since Hussein et al. (2009) have demonstrated that along with some NTM that could be viable but cultivable, there are some that cannot be cultured because of the unknown requirements for their growing in vitro.

Most of NTM species identified in this work included species that have been associated with human illness in Mexico and in other countries (Cassidy et al., 2009; Escobar-Escamilla et al., 2014). For example, ***M. chelonae***, ***M. abscessus*** and ***M. fortuitum*** have been associated with skin, soft tissue and pulmonary infections (Franco-Paredes et al., 2018; Piersimoni and Scarparo, 2009). ***M. mucogenicum*** has been involved in catheter-related infections, central nervous system diseases, and skin, soft tissue and respiratory infections (Adekambi, 2009). Since it has been reported that the public potable tap water supply in Mexico City harbors NTM (Perez-Martinez et al., 2013), we therefore suggest that this is the water that is the probable source of the NTM. Further work should be carried out in order to confirm this suspicion.

The elimination of mycobacteria from purified water is complicated mainly because of they are highly resistant to disinfectants and commonly used treatments in water purification systems and because of their capacity to form biofilms on various surfaces (Oriani et al., 2018). Interestingly, Tatchou-Nyamsi-Konig et al. (2009) have reported that ***M. avium*** can survive attached to polyethylene terephtalate (PET) water bottles, and that the cell adhesiveness to the PET wall increases with time.

Statistical analysis of our data showed a significant direct correlation between the presence of NTM and the presence of AMB and FC. Nevertheless, some previous analyses reported by us (Perez-Martinez et al., 2013) and other authors (Briancesco et al., 2010) found no correlation between the presence of NTM with aerobic mesophilic bacteria or with the number of coliforms or fecal coliforms in water samples. Therefore, further studies with a large number of purified water samples are required to corroborate this result.

5. Conclusions

This study showed that most of the purified bottled water analyzed had unsatisfactory microbiological quality and some harbored NTM associated with illness. Hence, authorities need to enforce the legislation that establishes the sanitary specifications of purified bottled water in order to reduce the risk of transmission of gastrointestinal diseases and others that are a consequence of their consumption. Moreover, our results could help Mexican health authorities intensify efforts in routine monitoring of activities in the purified bottled water industry with a view to supply safe and healthy water to the public.

Declaration of Competing Interest

None.

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