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Prevalence of *Legionella* species in hot water of Moorish baths "Hammams" and domestic bathrooms in Oujda city, Morocco.

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Abstract

Turkish bath or Moorish bath has a worldwide reputation and it is widely frequented by Moroccans. This study was designed to determine, for the first time, the prevalence of colonization of *Legionella* species (*L. spp.)* in hot water collected from 51 Moorish baths "Hammams" and 11 showers of private apartments in Oujda city in the east of Morocco. Water sample analysis and *Legionella* identification was carried out according to the French *Legionella spp.* standard methods (AFNOR NF T90-431). Results showed that hot water from 37 Moorish baths (72.5%) contains *L. spp*. Twenty two baths (59.5%) of the positive baths were colonized by *L. pneumpohila* including the serogroup 1 (32.4%), and sergoups 2-14 (56.7%). Fifteen positive baths (40.5%) were contaminated by *Legionella* species other than *L. pneumophila*. The colony count of *L. pneumophila* is less than 10^3 CFU/L, and remained below the tolerated threshold (a maximum of 990 CFU/L is reached in one bath). Seven domestic bathrooms (63.6%) were *L. spp.* positive. The most frequently isolated specie was *L. pneumophila* (71.4%), with most isolates belonging to serogroup 1 and 2-14 (4 baths /7 and 5 baths /7 respectively). A single water sample yielded a maximum colony count of 990 CFU/L of *L. pneumophila*. These findings show that hot water of Moorish baths and domestics showers are contaminated by *Legionella*. Although the degree of contamination did not reach the threshold level of infection, a constant monitoring of water should be put in place to control the risk of infection.

1. Introduction

The environments that surround us are diversified sources of exposure to microbial pathogens, some, like *Legionella* bacteria, are a potential threat to human health with major economic consequences [1].

Legionella species are opportunistic bacteria of public health concern. They are small Gram-negative bacilli belonging to the genus *Legionella* and develop mainly in the alveolar macrophages [2, 3].

Legionella spp. are found worldwide. The major reservoirs for these bacteria are natural water and man-made aquatic environments [4]. Other optimal growth conditions are found in water systems in hospitals, hotels, cooling towers, homes, factories, showerheads, baths, spa pools, artificial fountains, and other sources of water mist [5-9]. Stagnant water and warm water temperature (between 20°C to 50°C) can promote the growth of *Legionella* and formation of solid organized communities named biofilms by adhering onto surfaces [10, 11].

The major risk factor for acquisition of *Legionella* is exposure to contaminated water sources by inhalation of the aerosol or aspiration of water contaminated with *Legionella* [12, 13].

The genus Legionella is associated with *Legionellosis*, collective term including the pneumonic and non-pneumonic forms of infection with *Legionella.* The *legionellosis* can present either as a mild febrile illness (Pontiac fever) or a potentially fatal pneumonia, *Legionnaires'* disease (*LD*). Everyone is susceptible to infection, but the risk increases with age, illness, smoking and compromised immune system with chronic diseases. Other groups at increased risk of infection include hospital and industrial plants workers, and frequent travelers frequenting hotels [14-16].

Currently, the *Legionella* genus includes 54 species and more than 70 different serogroups. More than 23 species have been proven to be responsible of *LD* [17-19]. *L. pneumophila* species are the major cause of diagnosed cases of *LD* (about 80% – 90%), including from 60% to 90% of the most virulent *L. pneumophila* belonging to serogroup (sg) 1. So far, at least 15 serogroups of *L. pneumophila* have been identified. The serogroups other than *L. pneumophila* sg 1 (mainly sg 4 and 6) are involved in about 20% to 30% of infections [6, 20].

LD is a notifiable disease in Europe, and surveillance coordinated by the *European Legionnaires' Disease Surveillance Network (ELDSNet)* of the *European Center for Disease Prevention and Control (ECDC)* reported that the notification rate of *LD* in the EU/EEA in 2013 was increased to 11.4 cases per million population compared to 9.7 cases/million population in 2011 [21]. *L. pneumophila* sg 1 was responsible for more than 85% of the cases *LD* [20]. The *Center for Disease Control and Prevention* recorded 4202 cases across the United States in 2011 (incidence rate of 1.36 cases per 100 000) [22].

Since the discovery of *LD* in Philadelphia in 1977 [2], the incidence has remained undervalued in Morocco. Environmental investigations, made in different areas in Morocco between 2008 and 2012, to evaluate the importance of *L. pneumophila* contamination of production networks and distribution of domestic hot water systems, reported a mean frequency of isolation of 32%. The prevalence of *L. pneumophila* exceeding the acceptable level of 10^3 CFU/L varied between 62% to 67% [23, 24]. Although Moroccans epidemiological data do not report cases of *LD*, an epidemiological survey, conducted over four years by the Pasteur Institute of Morocco, revealed two sporadic cases of *Legionellosis* in 2008 and a group in 2011 [25].

The Moorish bath, "Hammam", or Turkish bath, is a public bathing retreat with hot and humid environment. It is an incredibly important part of Moroccan social culture and life and is still the usual manner of cleaning the skin and body for every social class, in spite the modernization of home bathrooms.

Few studies have been carried out in order to establish a health risk analysis of cold and hot water related to the presence of microbial pathogens in the Moroccan Moorish baths. The contamination with a number of pathogenic bacteria (such as *E. coli*, *P. aeruginosa* and *S. aureus*) has been reported in some Moorish baths in cities such as Rabat and Marrakech [26, 27]. So far, no studies have been performed to assess the colonization of *Legionella* in hot water of Moroccan Moorish baths. The present study was undertaken to determine, for the first time, the prevalence of *Legionella ssp.* in hot water samples collected from Moorish baths and in some private bathrooms in Oujda city located in the east of Morocco.

2. Materials and methods

2.1. Sample collection

A total of 51 Moorish public baths and 11 bathrooms of private apartments in Oujda city eastern Morocco, (4°41'12"N 1°54'41"W), were investigated for the occurrence of *Legionella spp*. The study was performed in the analytical control quality laboratory at the university Mohamed First, Oujda, during January to July 2013.

Hot water samples from each bath were collected from showerheads or bath taps in 500 milliliters sterile containers after a flow of 2-3 min to eliminate any cold water inside the tape or flexible pipe. Before sampling, a sterile swab was inserted into faucet outlets to dislodge the sediment. The swab was agitated vigorously on a vortex in 2 mL of sterile distilled water in order to re-suspend the sediment from the swab to the aliquot. The resulting samples were kept at 4°C and all analyses were performed within 12-hours.

2.2. Bacteriological analysis

The microbiological culture and quantification of *Legionella spp*. and *L. pneumophila* was conducted regarding to the conventional method described in the French standard "AFNOR NF T90-431", which conforms to international standard method ISO 11731. Five hundred mL of the water samples were concentrated by filtration through a 0.22 µm filter membrane (Satorius AG, Goettingen, Germany), and the membranes were re-suspended in 5 mL of sterile distilled water. A ten-fold dilution was made from this re-suspension in sterile water. A portion of this suspension was subjected to standard heat and acid treatments: a 2 mL of the concentrated portion was heated in 50°C water bath for 30 minutes in order to reduce the number of other micro-organisms before culture. Another 2 mL of the concentrate was mixed with 2 mL of 0.2 mol/L HCL-KCl buffer (pH 2.2) and kept at room temperature for 5 min.

Aliquots of 0.1 ml of the original and concentrated samples (with and without heat pretreatment, and 1:10 diluted or not), and 0.2 mL of aliquots acid treatments were plated onto selective GVPC agar, a modified BCYE agar (buffered charcoal yeast extract) -containing glycine (3 g/L), vancomycin (1 mg/L), polymyxin B (80,000

UI/L) and cycloheximide (80 mg/L). The plates were incubated at 36°C +/- 2°C in a humidified atmosphere for 10 days. To confirm the identification of the *Legionella* species, collected colonies with the typical ground glass appearance of *Legionella* species were sub-cultured onto BCYE agar with L-cysteine or without L-cysteine. The colonies grown only on BCYE agar with L-cysteine were selected and identified as *Legionella* on the basis of serological features.

Cysteine dependent colonies were used for species and/or serogroup determination by a commercially available latex slide agglutination test (Oxoid Legionella Latex Test, DR0800M, OXOID Limited, UK). This test allowed a separate identification of *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2-14 and detection of seven other *Legionella* species which have been implicated in human disease: *Legionella longbeachae* 1 and 2, *Legionella bozemanii* 1 and 2, *Legionella dumoffii, Legionella gormanii, Legionella jordanis Legionella micdade and Legionella anisa.*

3. Results and Discussion

Due to the health risk of infection by *Legionella*, all artificial water environments which could be potentially colonized by these microorganisms should be regularly monitored. Current regulatory and management guidelines for the control of these organisms are informed by risk assessments [28].

Moorish baths are characterized by ideal humidity and temperatures conditions for *Legionella* growth. These public baths are widely used by Moroccans for bathing, and they are still so popular due to beneficial effects on skin and health. For this reason, water must therefore respond to specific microbiological quality and safety requirement for bathing. However, there is no published information on the contamination by *Legionella* of water supply systems in these public baths. Therefore, the aim of this study was to appreciate the potential pollution of hot water (used by the public for bathing in Moorish baths) by *Legionella*. In parallel, hot water samples from some domestic baths were also subjected to analysis for *Legionella*.

Among 51 Moorish baths of Oujda city, *Legionella* species were detected in 37 baths, which accounted for 73% (Table 1). Different areas of the city were contaminated, and the highest concentration of positive baths was found in the center of the city (12 baths (23.5%)), whereas the area N°5 showed the lowest contamination (5.9%).

Table 1: Frequency of isolation of *Legionella spp.* from the hot water of Moorish public baths and private baths by location in Oujda city.

Location	Public Moorish bath		Private bath	
	No. of bath	No. of Legionella positive (%)	No. of bath	No. of Legionella positive (%)
1	7	7 (13.7)	5	5 (45.4)
2	12	12 (23.5)	0	NA
3	9	8 (15.7)	4	1 (9.1)
4	10	7 (13.7)	2	1 (9.1)
5	13	3 (5.9)	0	NA
Total	**51**	**37 (72 .5)**	**11**	**7 (63.6)**

NA: Not Applicable.

The distribution of *Legionella* cell numbers (colony-forming-units (CFU/liter)) is shown in Table 2. According to these results, *Legionella* was isolated in 30/51 public baths over 1000 CFU/liter (59%). It is worth to note that in 3 baths the enumeration exceeded 5.10^3CFU/liter and that approximately a quarter (14/51 (27.4%)) of water samples yielded *Legionella* count of ≤ 250 CFU/liter.

The serological distribution of species and serogroups of *Legionella* was summarized in Table 3. Results showed that *L. pneumophila* were the most abundant and accounted for 59.5% of all isolates in public baths, whereas *Legionella* species other than *L. pneumophila* accounted for 40.5% of the total. Among *L. pneumophila* species, *L. pneumophila* serogroups 2-14 were the most frequently represented. They were isolated alone or together with *L. pneumophila* serogroup 1 from 21 public baths (56.7%). The most virulent strain – *L. pneumophila* sg 1 was recovered from 32.4% of *L.* positive baths.

Table 2: Concentration of *Legionella spp.* isolated by colony count from hot water samples from Moorish public baths and private baths.

CFU/L	Public baths No (%)	Private baths No (%)
Geometric mean	1116.2	1112
Median	1350	1935
Min-Max	5 - 5625	10 - 4095
≤ 250	14 (27.4)	4 (36.4)
250 - 10^3	7 (13.7)	0 (0)
10^3 - 4. 10^3	24 (47.1)	5 (45.4)
> 4. 10^3	6 (11.8)	2 (18.2)

Globally, the average colony count of bacteria belonging to *L. pneumophila* was low (mean: 305.5 ± 122.9 CFU/liter, median: 249 CFU/liter), and all baths did not exceeded the acceptable level of 10^3 CFU/liter as recommended by the regulations. However, in one bath, *L. pneumophila* concentration was 990 CFU/liter, which is close to the threshold level of infection.

Table 3: Comparative distribution of *Legionella* species between 44 *Legionella* positive water from Moorish public baths and private bathrooms.

	***L.* positive public baths (n = 37)**	***L.* positive private baths (n = 7)**	**Total (n = 44)**
	No. (%) of *Legionella* isolates		
Non *L. pneumophila*	15 (40.5)	2 (28.6)	17 (38.6)
L. pneumophila (L.p.)	22 (59.5)	5 (71.4)	27 (61.4)
L. p. sg 1 and *sg 2-14*	11(29.7)	4 (57.1)	15 (34.1)
L. p. sg 1 alone	1 (2.7)	0 (0)	1 (2.3)
L. p. sg 2-14 alone	10 (27)	1 (14.3)	11 (25)

L. p.: Legionella pneumophila

In the residential bathrooms, there is also evidence of a widespread diffusion of the bacteria in domestic hot water taken from shower headers. Among 11 private baths examined, 7 were contaminated by *Legionella spp.* (63.6%, Table 1) and the mean number of *legionella* was 1.11 x 10^3 CFU/liter. Almost half of examined baths ranged from 10^3 to 4.10^3 CFU/liter, and 2 baths contained $\geq 10^4$ CFU/liter (Table 2). *L. pneumophila*, which is highly pathogenic for man and causes *Legionnaires*'disease, was the most frequently isolated species (71.4%) compared to non *L. pneumophila* (28.6%). *Legionella* positive baths were colonized by *L. pneumophila* serogroups 1 and 2-14 almost at the same ratio. Concentrations of *L. pneumophila* remained below the threshold of 10^3 CFU/liter (mean 315.5 ± 222.8 CFU/liter, median 249 CFU/liter). The highest concentration of 990 CFU/liter was reached again in only one private bath colonized by both serogroup 1 and 2-14.

The detection of *Legionella* in hot water samples taken from our tested baths indicates that hot water supply system could be a potential source of infection, and points to several environmental risk factors regarding growth of *Legionella* in this system. Indeed, all of the Moorish baths took their water supply directly from untreated groundwater. The cleanliness of this type of water remained uncertain in the absence of regulatory measures and monitoring of the water quality. To answer some of these uncertainties, a previous water quality measurement in Moroccan Hammams served by wells in other area, found significant pathogen contamination including fecal indicators (*E. coli*, intestinal enterococci), *P. aeruginosa*, *S. aureus*, etc. [26]. However, no testing on *Legionella* has been reported. On the other hand, the findings available from several studies in other countries showed that *Legionella* are widespread in groundwater samples [29-31]). The sole study found in the literature related to *Legionella* prevalence in hot water from Turkish baths (Hammams) is that of Erdogan and Arslan (2015) [32]. They identified 21.2% of *Legionella* positive baths in Turkish baths in hotels in Alanya (Turkey). The most frequently encountered specie was *L. pneumophila* with high degree of contamination

ranging from 100 to > 1000 CFU/100 mL in 9 positive samples. Their results suggest that hot water systems in Turkish baths present a significant potential source of travel-associated *Legionnaires*'disease.
During this investigation, it was shown that most of contaminated Moorish baths were concentrated in the center of town, (Figure 1), and this is probably due to the buildings old age and/or failure in maintenance of the water distribution systems. This provides favorable conditions for the proliferation of bacteria.

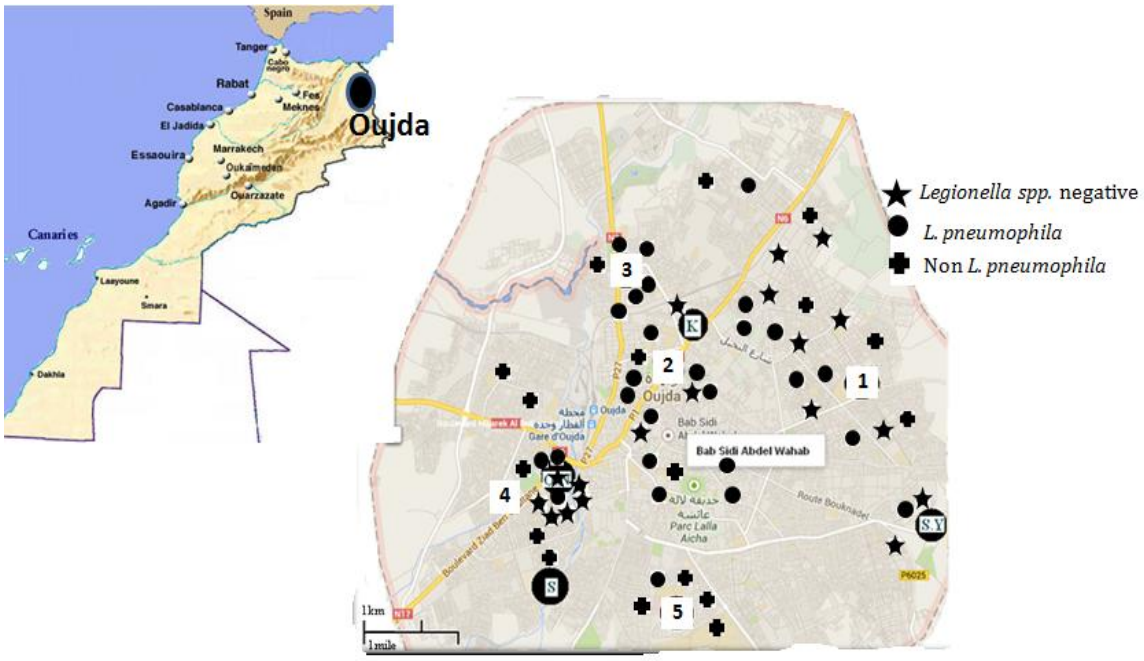

Figure 1: Distribution of *Legionella* species in the 62 investigated baths (Hammams and private baths) in Oujda city.

The ability of *L. pneumophila* to colonize domestic bathing facilities has been identified as a primary cause of outbreaks of Legionnaire's disease in humans [33, 34]. Previous microbiological studies of showerhead biofilms have traced *L. pneumophila* infection in hospitalized patients to bacteria in their home showers [35-37]. In fact, the showerhead environment provides an important interface for human infection through aerosolization and/or direct contact with *Legionella*. The inside of a showerhead forms a favorable niche for a cohort of microorganisms [38, 39]. Chlorination seems to be not effective at preventing colonization, in reference to a study conducted in UK reporting on the persistence of *L. pneumophila* sg1 in a domestic shower system despite repeated cycles of chlorination [33].
Our results showed that the examined showerheads are colonized by *Legionella spp*. and *L. pneumophila* is by far the most abundant species but remained at levels below limits tolerated by the regulation. These concentrations were much lower than those reported in the literature with maximum counts of 10^4 to 10^5 CFU/L, suggesting that showering seems to present a limited risk of acquiring *Legionnaires' disease* in our conditions [40, 41]. Furthermore, the concentrations found in our study are lower than levels reported by other *Legionella* surveys conducted on hot water distribution systems of hotels, hospitals and factories in different area of Morocco [24, 42].
Low or no *L. pneumophila* rates have been reported in other surveys of private bathroom outlets. Using a culture-independent methodology based on ribosomal RNA gene sequences to identify the composition of microbial populations associated with showerheads in a wide geographical area of U.S., Feazel *et al.* (2009) [43] found that the opportunistic pathogen *L. pneumophila* was encountered rarely in their survey. However, they found a complex and enriched microbial assemblage including *Mycobacterium spp*., *Sphingomonas spp*. *Escherichia spp.* and others. They concluded that the detection of high levels of non-tuberculous mycobacterium points to showerheads as source of opportunistic pathogens known for pulmonary disease. A similar study conducted in residences of a Korean city, reported failure to detect *Legionella* in showerheads but the predominance of numerous microorganisms, recognized as opportunistic and potential human pathogens in causing nosocomial infection [44].

Conclusion

This study, which reports the presence of *Legionella* in our region of eastern Morocco, extends the list of the possible sources of infection, including Moorish baths and private bathrooms among the contaminated sites. Although the critical thresholds of infection are not reached yet, vigilance for any microbial degradation of water must be maintained. In these premises, the main source of water is untreated groundwater whose microbial quality is uncertain. It is important that relevant authorities should be involved to ensure adequate measures are in place to control the risks. An important part of many *Legionella* control regimes is inspection and regular maintenance by cleaning and disinfecting the water system periodically. Chlorine dioxide represents the best choice for reducing the risk of *Legionella* transmission, although it does not eradicate it. Other control methods include copper-silver ionization. To ensure that they remain effective, their application will need suitable assessment as part of the overall water treatment program.

This study should be conducted to include other cities and a substantial number of Moorish and domestic baths. A better understanding of the potential public health impact of *Legionella* colonization could help in the development of control strategies for prevention of *legionellosis*.

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