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Investigation of *Legionella Pneumophila* in Hot Water Systems in Morocco

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ABSTRACT

Legionnaires' disease, the pneumonic form of legionellosis, usually is acquired by inhalation or aspiration of *Legionella* from contaminated environmental sources. Potable water is an important source of both nosocomial and community acquired *Legionella* infections. The species of *Legionella* are widely distributed in water environments. *Legionella pneumophila* is a relatively common cause of pneumonia. In patients with community-acquired pneumonia, the incidence ranges from 2 -15%. *Legionella pneumophila* serogroups were differently distributed according to heater type, water temperature, suggesting that *Legionella* strains may have a different sensibility and resistance to environmental factors and different ecologic niches. The aim of the present study was to determine the prevalence colonization of *Legionella pneumophila* in hot water distribution systems of hospitals and hotels and factories in Morocco and to examine the possible association with bacterial contamination and physic chemical parameters.

Keywords: *Legionella pneumophila, hot water distribution system, quality parameters, Morocco*

1. INTRODUCTION

Pneumonia is an important cause of morbidity and mortality. Legionellosis, or human disease caused by *Legionella* spp., can be community acquired [1-2], hospital acquired [3] and travel associated [4]. *Legionella* is an opportunistic pathogen with widespread distribution in the environment. The history of *Legionella* began in 1976, when a large outbreak of severe pneumonic illness occurred among visitors to the Legion congres in Philadelphia, USA. In 1977 the causal microorganism was isolated and named *Legionella pneumophila*. Numerous reports have demonstrated that the major sources for Legionnaires' disease are the hot water systems of large buildings including hospitals, nursing homes, and hotels [5]. *Legionella* is a common cause of hospital-acquired pneumonia, where patients may be at a higher risk for a severe infection [6]. Currently, the *Legionella* genus includes 54 species [7] and more than 70 different serogroups, and more than 23 species have been proven to be causative agents of Legionnaires' disease [8]. Most human infections are caused by *Legionella pneumophila* [9], and the predominant serogroup is serogroup 1 [10]. Approximately 75% of *Legionella* infections are caused by *Legionella pneumophila* serogroup 1, whereas 20-30% is caused by other serogroups of *Legionella pneumophila*, and 5-10% by different *Legionella* species [11]. *Legionella* are Gram-negative bacteria, which are able to reproduce at temperatures between 25 and 45°C [12] and survive in temperatures of up to 60°C [13]; therefore, they are ubiquitous in both natural and man-made environments and are frequently found in domestic water systems [14-15]. A better understanding of the risk factors for *Legionella* colonization in artificial water systems could help in the development of control strategies for prevention of legionellosis. For instance, *Legionella* contamination of domestic hot water was found to be associated with a centralized system, a greater distance from the heat source. From these environments, the bacteria can be transmitted to humans by inhalation of contaminated droplets. Inhaling of droplets (1-5 µm) containing aerosolized organisms is a common cause of infection and carries a mortality rate of 10% to 30% [16]. The objectives of this study were to investigate the prevalence of *Legionella pneumophila* in hot water distribution systems of hospitals, hotels and factories in Morocco, their possible association with bacterial contamination, and the physic-chemical characteristics of the water (pH, temperature, conductivity and Turbidity).

2. MATERIALS AND METHODS

2.1 Sample Collection

An analysis was carried out of 54 water samples were collected from 22 hotels and 15 from public hospitals, 59 samples from the factories that are representative of different Moroccan regions (northern, central, and southern). At the time of sampling a questionnaire was used to obtain details of the water heating system: the type of distribution (centralized or independent, gas or electrically heated), the volume of the reservoir tank (if present) and the distance of the boiler from the sampling point. Hot water samples were collected from bathroom outlets (showerheads or bath taps) in sterile bottles. In order to neutralize residual free chlorine, sodium thiosulfate was added to sterile bottles for bacteriological analysis.

2.2 Microbiological Analysis

Microbiological samples were analysed within 12 hours of collection using the standards methods. In order to determine of water bacteriological quality according to standard Methods, The detection and enumeration of total coliforms and fecal coliforms according to standard NF T 90-414; 100 ml water samples were filtered through 0.45 µm pore size membranes (Sartorius AC, Goettingen, Germany), were enumerated on Lactose TTC agar with tergitol 7 medium (Biokar) at 37°C and 44°C for 24 to 48 h. The detection and enumeration of fecal enterococci in water was done by a membrane filter procedure according to standard ISO 7899-2; 100 ml water samples were filtered through cellulose nitrate membrane filters with 0.45 mm pore size; the fecal streptococci were enumerated on Slanetz and Bartley medium (Oxoid) at 37°C for 48 h and confirmed with membranes were placed on Bile Esculin Azide Agar (Merck) and incubated for 24 h at 37°C. For detection and enumeration for the spores of sulfite-reducing anaerobes, 100 ml water samples were filtered and enumerated on Tryptose Sulfite Cycloserine Agar (Biokar) at 37°C for 48 h according to standard ISO6461-2. *Pseudomonas* spp were enumerated on Cetrimide agar (Biokar) at 37°C for 48 h, the colonies grown on *Pseudomonas* agar were then subcultured and identified using the API 20NE commercial kit (bio-Mérieux, Marcy l'Etoile, France) according to standard NF T 90-419. The total microbial counts at 36°C and 22°C were determined twice by the method on plate count agar (Oxoid) plates were incubated at 36°C for 48 h or at 22°C for 72 h according to standard ISO6222.

Culture and identification of *Legionella pneumophila* were carried out by using the NF T 90-413 method. One liter of a water sample was concentrated by membrane filtration 0.2 µm (Sartorius AC, Goettingen, Germany). The filters were placed in 5 ml of sterile water and sonicated for 2 min (Fischer Bioblock scientific sonicator; Ilkirch, France) at 60 kHz. All samples were subjected to standard heat and acid treatments. A 2 ml portion of this concentrate was placed in a 50°C water bath for 30 min for the decontamination of the microorganisms. 2 ml of 0.2 mol/l HCl-KCl buffer (pH 2.2) was mixed to 2 ml the aliquot of the concentrate for 5 minutes at room temperature. Aliquots of 100 µl of the concentrated samples (with and without heat treatment) and 200µl of Aliquots acid treatments were spread plates of GVPC (AES CHEMUNEX).The plates were incubated at 36°C± 2°C, in a humidified environment with 2.5% CO2 colonies were counted after 3, 5, and 10 days. Colonies morphologically consistent with *Legionella* spp. were placed on to buffered charcoal yeast extract (BCYE with cysteine) agar (Oxoid) and blood agar (Oxoid), and incubated for 48 h. Colonies growing on BCYE agar but not on blood agar were definitively identified as *Legionella* spp. using a commercially available latex agglutination test (Slidex *Legionella*- kit, BioMérieux) that distinguishes *Legionella pneumophila* serogroup 1, *Legionella pneumophila* serogroups 2 to 15 (polyvalent), and *Legionella anisa*.

2.3 Physical and Chemical Analysis

Water temperature was measured in situ using a portable pH meter, for pH was measured by pHmeter (HI 9321 microprocessor, Hanna, portigal). Standard techniques were used to measure water conductivity (conductivitimeter Consort C 830) and water Turbidity (2100 turbidimeter HACH).

2.4 Statistical Analysis

Statistical tests were conducted to examine the association of the incidence of *Legionella pneumophila* with water quality parameters. The incidence of this organism was regarded as a binary random variable (present or absent) and related to the level of a factor (such as temperature, pH, Conductivity, turbidity and plate count at 36 and 22°C) that were divided into a set of equal, non-overlapping intervals. The results were treated by correlation analysis; performing calculations were performed using XLSTAT Software version 2012.4.02. Pearson correlation coefficient (r) was used to show correlation between *Legionella.*

3. RESULTATS

3.1 Frequency of Legionella Occurrence and Species Identification in Hot Water Potable Distribution

A total of 128 samples collected from hotels (n=22), Hospitals (n=5) and factories (n=12) were examined for the presence of *Legionella* (Table 1). *Legionella pneumophila* was detected in 19.5% of all samples in the hot water potable of different areas in Morocco.

The results of 15 hot water samples were collected from five hospital units, only 1 (6.6%) was found to be contaminated by *Legionella pneumophila*. for 59 samples water of factories 1 (1.7%) was colonized by *Legionella pneumophila*. A total of 22 hotels and 54 water samples (42.6%) were contaminated *by Legionella pneumophila*.

Of the total of 25 isolates in the hot water potable distribution of different areas in Morocco, *Legionella pneumophila* serogroup 2-15 was the most frequently isolated species (52% of the isolates), while 36% of the positive samples contained $\geq 10^3$ CFU L^{-1} *Legionella pneumophila* and 8% $\geq 10^4$ CFU $^{-1}$ (Table1).

Among the positive hotels, six were colonized by *Legionella pneumophila* serogroup1 and five were colonized by serogroup 2-15. For hospitals and factories one of each structure were colonized by *Legionella pneumophila* serogroup 1 (table 1).

Table 1: Characteristic of *Legionella pneumophila* contamination in hot water samples examined (% of positive simples)

	Hotels					Hospitals					Factories				
	+ve	with X≤250	with 250 ≤X≤ 10^3	with 10^3 ≤X≤ 10^4	with 10^4≤ X	+ve	with X≤250	with 250 ≤X≤ 10^3	with 10^3 ≤X≤ 10^4	with 10^4≤ X	+ve	with X≤ 250	with 250 ≤X≤ 10^3	with 10^3 ≤X≤ 10^4	with 10^4≤X
LP sg 1	10/25	1/10 (10%)	2/10 (20%)	6/10 (60%)	1/10 (8.3%)	1/25	1/1 (100%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	1/25	1/1 (100%)	0/1 (0%)	00/1 (0%)	0/1 (0%)
LP sg 2-15	13/25	2/13 (15.4%)	0/13 (0%)	9/13 (69.2%)	2/13 (15.4%)	0/25	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/25	0/ (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)

The presence of *Legionella pneumophila* in hot water potable distribution was detected according to the type of water heating system (centralized or independent). A higher level of *Legionella pneumophila* colonization was found in the water of centralized systems. About 50% of centrally heated hotels contained *Legionella pneumophila* (range: 200-59000 CFU L^{-1}), of which 60% $\geq 10^3$ CFU L^{-1} and 8.3 % $\geq 10^4$ CFU L^{-1} were *L. pneumophila* serogroup 1, while 69,4% of the positive samples contained $\geq 10^3$ CFU L^{-1} and 15,4% $\geq 10^4$ CFU L^{-1} *L. pneumophila* serogroup 2-15. For others structures (hospitals and Factories) *Legionella pneumophila* were demonstrated a load $\leq 10^3$ CFU L^{-1}.

3.2 Correlation of the Occurrence of *Legionella* with Water Quality Parameters

Various water quality parameters were examined statistically by Pearson's correlation for their association with the presence *Legionella pneumophila*. The microbiological parameters of hot potable water distribution examined were total coliforms and fecal coliforms, fecal streptococci, the spores of sulfite-reducing anaerobes , *Pseudomonas* spp and amoeba were proved in our study not associated with *Legionella* contamination, while water samples contaminated by *Legionella pneumophila* was associated with the total microbial counts at 36°C and 22°C. Bacterial load was variable ranging from 0 to 180 CFU/ml and from 0 to 88 CFU/ml at 22 and 36°C, respectively, demonstrated association between *Legionella* spp. growth in water samples. The relationship between microorganisms (total microbial counts at 36°C and 22°C), and *Legionella* is shown in tables 2.

Table 2: Pearson's correlation coefficients total microbial counts at 36°C and 22°C with *Legionella*

Variables	*Legionella*	TC at 22°C	TC at 36°C
Legionella	1	0,553	0,37
TC at 22°C	0,553	1	0,881
TC at 36°C	0,37	0,881	1

Bold values are different from 0 at a significance level of alpha = 0.05

Pearson's correlation coefficients showed that bacterial counts have a positive correlation with *Legionella* (r=+0.370 and r=+0.553, P<0.05) for respectively total microbial counts at 36°C and 22°C. This result proved that there is statistically significant difference between the presence of *Legionella* and total microbial counts at 36°C and 22°C.

Legionella presence was compared with water temperature levels. The temperatures were divided into five categories: 20–30, 30–40, 40–45, 45–50 and 50-56°C. Most samples were high water temperature (>30°C). The highest incidence of *Legionella* was found in the range of 30–40 (28.5%), followed by 40–45°C (72.7%), and 45–50 (19.4%), whereas none of 20–30 and 50-56°C. In our study *Legionella pneumophila* was present between 39°C to 50°C. Regarding the Chemical characteristics of the hot potable water appeared to be related with *Legionell*a presence. In this study, the incidences of *Legionella* in water samples with pH ranges

of 6-7, 7-8 and 8-9 were 69.2% (9/13), 2.3% (15/57), and 1,7% (1/58), respectively. Pearson's correlation coefficients showed water temperature and pH have a negative correlation with *Legionella* (r = -0.362 and r= -0.874 respectively, $P<0.05$) (Table 3). Samples positive for *Legionella* species ranged in conductivity from 489-3960 µs/cm and 0.64-2.9 NTU for turbidity.

Table 3: Pearson's correlation coefficients physical and chemical parameters with *Legionella*

Variables	*Legionella*	T (°C)	pH at 20°C
Legionella	1	-0,362	-0,487
T (°C)	-0,362	1	0,394
pH at 20°C	-0,487	0,394	1

Bold values are different from 0 at a significance level of alpha = 0.05

4. DISCUSSION

Legionella is an ubiquitous bacterium that could create problems for public health; most cases of Legionnaire's disease are sporadic but nosocomial or community outbreaks can occur, such as those related to tourist accommodations [17] The prevalence of *Legionella pneumophila*, the causative agent of Legionnaire's disease, in nature and man-made aquatic environments has been documented in literature [14-15]. The presence of *Legionella pneumophila* was detected with a high frequency and at high levels in the hot potable water distribution in Morocco. Our data indicate that at the time of this study, 19.5% of the hot potable water distribution in Morocco was positive for *Legionella pneumophila*. In previous studies has demonstrated *Legionella* contamination ranging from 31.5% [18] to 32.5% [19]. The concentrations of this species reached value $\geq 10^3$ $CFUL^{-1}$ in 60% of the samples positives and $\geq 10^4$ $CFUL^{-1}$ in 12%, while in the Morocco studies 38% were $\geq 10^3$ CFU L^{-1} [19] and 45% [18]. The water samples obtained from the hot potable water distribution structures in this study provide information about the prevalence of *Legionella pneumophila* in hotels, hospitals and factories. *Legionella pneumophila* serogroup 1 and serogroup 2-15 was isolates in hotels from 18.5% and 24% respectively of the samples examined, which deserves special attention on the high frequency of *Legionella pneumophila* serogroups 2-15, which was isolated from 69.2% of the contaminated $\geq 10^3$ CFU L^{-1} and from the charge $\geq 10^4$ CFU L^{-1} in 15.4% over period the study. Moreover, *Legionella pneumophila* serogroup1 load was predominantly high ($\geq 10^4$ CFU L^{-1} in 8.3% or ranging from 10^3 to 10^4 CFU L^{-1} in 60%). Furthermore, in our study of hot water systems contamination in the hospitals and factories, *Legionella pneumophila* serogroup1 was found only in 100% positive samples on load $\leq 10^3$ CFU L^{-1}. However the concentrations found in this study not only exceed the threshold of risk estimated by several authors but are also higher than the safety levels established in the regulations now in force in many European Countries regarding the control of legionellosis.

Worldwide, *Legionella pneumophila* serogroup 1 is the most common agent of Legionnaires' disease, accounting for about 80 to 90% of the reported cases [20, 21-21] and approximately 70% of the European travel-associated cases [22]. In contrast, *L. pneumophila* serogroups 2-15, although accounting for more than 50% of the isolates obtained from man-made aquatic systems, account for only 15 to 20% of community cases. The discrepancy between environmental isolates and clinical expression of disease has been observed by Doleans et al. [23], who suggested that there are differences in virulence rather than greater abundance in water distribution systems.

Another aspect of the lowest risk of *Legionella* colonization was found in independently heating systems. This result could explain of relationship with distribution systems and frequency of water used in structures, because the contaminating organism (*Legionella pneumophila* serogroup 1 and *Legionella pneumophila* serogroups 2–15) was specific to a system (centralized or independent heaters) [6]. Our hypothesis is that *Legionella* strains substantially differ in their sensitivity to environmental risk factors and, as a consequence, may have different ecologic niches. *Legionella pneumophila* responsible of Legionnaires'disease cases [21] was predominantly isolated from centralized water heating systems, despite the fact that they were more frequently contaminated. The higher levels of *Legionella* in centralized water heating systems can be explained by the stagnation of the water in the storage tanks and within any closed pipes; such stagnation enhances the formation of biofilm. Only independent heaters, ones appeared to be most protective against contamination by *Legionella pneumophila* [13]. This suggests that the contamination by *Legionella pneumophila* was associated with water quality parameters. This association may be explained by presence in contamination distribution systems of biofilms consisting of bacteria and other microorganisms embedded in protective layer with entrained debris attached to a surface [24]. The total plate count at 22 and 36°C could be used as an indicator of the presence of *Legionella*.

It is known that *Legionella* easily reproduces in high temperature in hot water systems. Zanetti et al. [25] reported that there was an inverse association between water temperature and the concentration of *Legionella pneumophila*. However, Leoni et al. [26] found that no *Legionella* species were present at water temperatures above 43°C. Ohno et al. [27] and Brooks

et al. [28] found that likelihood of successfully culturing *Legionella* declined with increasing temperature. In our study, the temperature and pH were correlated negatively with the counts of *Legionella pneumophila* serogroup 1 and of *Legionella pneumophila* serogroups 2-15. Ohno et al. [27] found that the optimal pH for *Legionella* in an aquatic environment ranged from 6–8, which is in agreement with the results of the present study. These findings show the reverse other reports, which indicated a positive association of *Legionella pneumophila* with pH [29, 13-30]. The differences in distribution of species according to water characteristics confirm the hypothesis of other reports, which indicated that *Legionella pneumophila* differ in sensitivity to environmental risk factors and have different ecological niches [6-13].

5. CONCLUSION

Our observation suggest that *Legionella pneumophila* should be considered while examining environmental contamination, which is essential to evaluate environmental risk factors and select the most appropriate prevention and control measures [31]. The absence of a correct prevention of *Legionella* infections might cause a lot of legal and economic problems (deaths, negative impact on tourism and nosocomial disease, etc.). pH, temperature and total plate count at 22 and 36°C can be used as a indicator for the risk to the presence of *Legionella pneumophila* in hot potable water, there reasons for these trends are currently unknown, but our data suggest that *Legionella pneumophila* and serogroups may have different ecological niches and/or that their ability to survive in man-made water environments varies with the environmental conditions.

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