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Identification of Environmental Factors Involved in Legionella Development

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Abstract

Legionella are aerobic, gram-negative bacteria which grow in natural aquatic environments. Only half of the cases are elucidated, partly due to the fact that environmental factors involved in Legionella development are not well characterized. The purpose of the study is to identify environmental factors involved in legionella development. Secondary data recorded from 2000 to 2011 of Rennes, France were used for this study. Descriptive analysis of free chlorine, pH, iron, water temperature, total hardness and conductivity were Logistic regression was done to correlate the legionella and physical chemical parameters. The mean value of free chlorine, pH, iron, water temperature, total hardness and conductivity were found high in legionella positive samples except nitrate. Total hardness of water ($p<0.417$), nitrate ($p<0.449$) and iron ($p<0.645$) were not correlated with the presence of legionella. Adjustment was done for nitrate, total hardness and iron using multivariate logistic regression but there was no correlation with legionella. Therefore, various environmental factors such as free chlorine, pH, water temperature and conductivity are associated with the presence of legionella.

Key Words: Legionella species, Environmental factors, Water, France

Introduction

Legionella are rod or coccoid shaped which do not form spores. It is known that life cycle of legionella is divided into two stages. In the first phase, the bacteria are non motile and have a low virulence. It is called the replicative phase. Likewise, in the second phase, the bacteria are thicker and shorter. They have well developed flagella and are very virulent which is called as infectious phase[1]. Legionella species are everywhere in freshwater environments but can also be found in wet mud. However, the main environments for some species like *Legionella longbeachae* are soil and composting site[10].

The virulence of the legionella bacteria is developed from the parasitic interactions between amoebas and other eukaryotics. Likewise, the bacteria can be grown anywhere from 5 to 63 Celsius degrees, whereas, maximum growth occurs between 25 and 40 Celsius degrees. The *Legionella micdadei* and amoeba host have been found in highly acidic geothermal environments[9].

Legionnaires' disease is a severe pneumonia that can be accompanied by extrapulmonary manifestations, such as renal failure, encephalopathy, and pericarditis. The symptoms of Legionnaires' disease start with low fever, malaise, a mild cough, muscle aches and gastrointestinal symptoms. Similarly high fever, alveolitis and bronchiolitis are the later development of the disease[1]. The transmission between human beings has never been observed to this date. Therefore, it is focused on the elimination of legionella pathogens of hot water systems and cooling towers for the prevention and control of Legionellosis. Outbreaks occur usually from purpose-built water systems where temperatures are warm

enough to encourage growth of the bacteria, e.g. in cooling towers, whirlpool spas, hot water network and their topology, and water used for domestic purposes. This is due to symbiotic associations of legionella with amoebas[1].

Legionella was first recognized in 1976 as human pathogens after an outbreak of pneumonia among veterans attending a convention in Philadelphia. It was estimated that about 20,000 cases of Legionnaires' diseases were annually occurred but only 1000 cases were reported in the United States . The case fatality rate of legionnaires' disease can be increased with risk factors like alcoholism, cigarette smoking, age and cancer .

In France, almost 1500 cases of legionellosis are detected each year. The incidence rate of legionnaires' disease was about 1.9 per 100 000 population in 2009 . About fifty four sporadic isolates were gathered and seventeen epidemic isolates were found in recent five outbreaks in France. Forty nine endemic isolates designated Paris was isolated from various parts of France[1].

Factors involved in Legionella development

The necessary growth requirements for the development and protection of microbial communities as well as legionella are provided by biofilms. It is difficult to remove biofilms from the complex water distribution systems but the prevention of biofilms is necessary to control the proliferation of legionella bacteria[7].

The motility and virulence of the cultured *Legionella pneumophila* is also affected by temperature. More flagella have been assembled and legionella cells express additional proteins and flagellin RNA incubated at 30 °C than at 37 °C[7, 8]. Bacterial populations released in an aqueous environment are frequently exposed to stresses due to limitations and changes in nutrient availability, temperature, salinity, oxygen, and pH. Bacteria often enter a "temporarily non-cultivable stage. The HPCs at both 22 and 37 °C and concentrations of Zn, Fe, and Mn were positively correlated with legionella, while total hardness and temperature were negatively correlated . Growth of *Legionella species* are supported by the temperature of water, stagnation, flow, deterioration of pipe, materials used in pipe, flushing and water stresses[5,1], while water characteristics such as hardness and concentrations of chemical elements have been only proposed . The important factors that involved in *Legionella pneumophila* growth are iron, potassium and zinc in lower concentrations, while toxic in high minerals concentrations[5]. Similarly, the growth and development of Legionella pneumophila is inhibited by the appearance of copper in water networks[2].

It is difficult to control the growth of bacteria and prevention of disease caused by bacteria without knowing the environmental factors. Only half of the cases are elucidated, partly due to the fact that environmental factors involved in Legionella development are not well characterized. This research is important to identify such growth factors for the development of *Legionella species* and correlation between physical chemical parameters and *Legionella species* before and after the evolution of the water distribution system in Rennes in 2006. The general objective of the study was - to identify the environmental factors involved in legionella development in the city of Rennes.

Materials and Methods

Secondary sources of data from the database of Laboratoire d'Etude et de Recherche en Environnement et Santé (LERES), EHESP were used for this research. Water sources were from the cooling towers, swimming pools and water networks of Rennes, France, as the LERES carry out the regulatory surveillance of these sources for the presence of legionella. Likewise, laboratory diagnoses of water samples for physical chemical parameters and legionella were already performed and recorded as a database. It was the normal surveillance of water sources from 2000 to February 2011.

Statistical analysis was done by using STATA 11 version and geographical information system (ArcGIS). Descriptive statistics (mean, median, variance, CI and skewness) of physical chemical characteristics from the database have been

analysed and presented in the form of tables and graphs. Likewise, logistic regression analysis was done to correlate the presence of the legionella with the various physical and chemical parameters.

Correlation matrix was estimated to know the correlation between two or more independent variables. The multivariate logistic regression was computed based on the correlation matrix and R^2 from univariate logistic regression. Mainly, the univariate logistic regression was carried out because of the high positive correlation between independent variables.
The map of Rennes was used to point out the exact places of water sampling and the places, where positive legionella was found.

Results

Most of the parameters were either negatively or positively skewed from -0.09 to 13.42 (Table 1).
The average value of free chlorine for positive samples was 0.51 mg/l (0.38 to 0.64, 95 % CI) and 0.22 mg/l (0.17 to 0.27, 95 % CI) for negative samples. Likewise, the average value of pH (8.27), total hardness of water (14.11°f), water temperature (20.15°C), iron (36.04mg/l) and conductivity (796.5 microS/cm) were found in legionella positive samples, which were higher than that of legionella negative samples. However, more than 27 mg/l (25.23 to 29.02, 95% CI) average nitrate was found in legionella negative samples which was higher than in positive legionella samples (Table 2). The high 95% confidence interval in positive legionella samples showed that the distributions of data were more fluctuated. The highest percentage of legionella was found in 2007, which was about 22 % and lowest about 2% in 2008. Likewise, eighteen percent in 2006, 17 % in 2001, 15 % in 2009 and 14 % in 2004 was detected from different areas of Rennes.

According to univariate logistic regression analysis, free chlorine [OR 3.38, CI (1.93 - 5.91), p<0.0000], pH [OR 5.05, CI (2.55 – 10.03), p<0.0000], conductivity [OR 1.001, CI (1.001 – 1.002), p<0.0000] and water temperature [OR 1.06, CI (1.03 – 1.10), p<0.0001] were significantly positively associated with the presence of legionella at 5% significance level. However, total hardness of water [OR 1.04, CI (0.94 – 1.16), P<0.417], nitrate [OR 0.99, CI (0.96 – 1.02), p<0.449] and iron [OR 1.002, CI (0.99 – 1.01), p<0.645] were not correlated with the presence of legionella. Likewise, multivariate logistic regression was applied for total hardness of water [OR 0.96, CI (0.82 – 1.11), p<0.560], nitrate [OR 0.98, CI (0.96 – 1.02), p<0.498] and iron [OR 1.003, CI (0.99 – 1.01), p<0.595], which were also not significant to the legionella (Table 3).

Discussions and Conclusion

Legionella pneumophila were found in water samples where mean values of free chlorine, water temperature, PH, total hardness of water, iron and conductivity were higher than in legionella negative samples (Table 2). Likewise, nitrate was measured high in legionella negative water samples. However, in the literature similar result was found in the concentration of nitrate and legionella positive and negative samples in water systems[1]. The presence of legionella in high average free chlorine (0.51 mg/l) value was possibly due to the existence of *Legionella pneumophila*. Similarly, high average pH environment was favorable to the growth of legionella but disinfectant chlorine was supported by low pH. However, *Legionella pneumophila* and *Legionella pneumophila* serogroup 2 to 14 were found positive with increases in pH but negative with other *Legionella spp.*[5]. Our results for pH and legionella association were contrasted with the result of Katz and Hammel[3]. This might be because of their experimental research.
The results of this study prove the significant positive correlation between free chlorine [OR 3.38, CI (1.93 - 5.91), p<0.0000] and the presence of legionella. In another study, the concentration of free chlorine was found a major

parameter for high contamination as previously. *Legionella pneumophila* serogroup 1 was significantly correlated ($p<0.0008$) with free chlorine concentrations above 0.2 mg/l but not ($p<0.3$) for other Legionella spp.[5, 2].

Likewise, pH of water [OR 5.05, CI (2.55 – 10.03), $p<0.0000$] was significantly positively associated with the growth of legionella. Similarly, conductivity [OR 1.001, CI (1.001 – 1.002), $p<0.0000$] and water temperature [OR 1.06, CI (1.03 – 1.10), $p<0.0001$] were also highly positively related to the development of legionella. In contrary, pH and water temperature were only associated with the proliferation of legionella but not related to the occurrence of *legionella spp*. Legionella may be introduced by the presence of amoebae but it needs certain condition to multiply[8]. These factors such as temperature and pH are important for the multiplication of legionella[1].

Corrosion level of pipes, stagnation and flow of water, flushing and temperature of water are also associated with the growth of *Legionella spp.* while hardness of water and concentration of trace elements have been only recommended [10]. Recently, investigation of correlation between legionella colonization and heterotrophic bacteria were also started[6, 3].

In our study, iron [OR 1.002, CI (0.99 – 1.01), $P<0.645$] was not correlated with legionella growth. Iron (Fe) and zinc (Zn) used in plumbing systems are possibly the cause of corrosion of pipes and high concentration of iron and zinc in water systems. Metallic pipes (cast iron) are often used in Italy [3] but also in France. Likewise, development and growth of legionella are the effects of metals leaked from water tanks and pipes[5]. Iron is an important growth factor for legionella in laboratory and legionella infection. Similarly, a multicentric research in Italy recommended that the occurrence of metals and relationship with legionella in water distribution systems are possibly due to geographical variations and many water treatment centers and sources[3]. A recent study in Japan reported a positive correlation between iron and *Legionella spp.*, while copper (Cu) and Zinc (Zn) were not associated[6]. Experimental studies showed the growth of *Legionella pneumophila* was enhanced by concentrations of Fe and Zn whereas it became toxic in high concentrations [5].

Copper, silver and other bacteriological parameters were not included in our study because of high number of missing values. However, some studies showed that copper and silver have important role in the development of legionella and other pathogens in planktonic phase and biofilms[6]. Likewise, the pipes that made up of galvanized steels were less likely to be contaminated than copper pipes[1-2].

The reduction in concentrations of free chlorine and increased in concentrations of iron and HPC are due to stagnation of water which strongly supports the development of legionella. Prevention and control of legionella contamination and legionellosis might be achieved by preventing water stagnation in water systems.

The results of our study showed that the physical chemical parameters such as free chlorine, pH, water temperature and conductivity were associated with the growth of *Legionella spp*. in Rennes. However, total hardness of water was also correlated with legionella after the integration of water systems into one at the end of 2006. Previous studies have suggested more physical, chemical and bacteriological parameters than what it used in this research. Though, this study might be helpful to produce the water safety plan, prevention of legionella, diminishing of physical chemical parameters. It also suggests that the pipes should be checked time to time, maintenance of water treatment center and sources, use of tap water after few minutes of flushing (especially for showers in swimming pools) and planning of environmental sanitation and health education programmes for local populations. Likewise, maximum efforts were used to match the place and date because of separate samples for legionella, and physical chemical parameters. It should be necessary to test the legionella positive water samples, for physical chemical and biological parameters as well. Most of the physical chemical parameters (free chlorine, pH, water temperature and conductivity) which were studied in this research have significant positive correlation with legionella. Therefore, this study explained the environmental factors are responsible for the growth and development of *Legionella pneumophila*.

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Annex

Table 1 Descriptive statistics of different physical chemical parameters

S.N.	Parameters	Obs.	Median	Variance	Min.	Max.	Skewness	Unit
1	Iron	941	30	1648.18	0	326	2.77	mg/l
2	Water temperature	1021	16	30.28	5	65	1.39	°C
3	pH	1001	7.85	0.16	6.75	8.85	0.10	pH
4	Free Chlorine	998	0.1	0.08	0	2.5	4.33	mg/l
5	Total Chlorine	642	0.1	0.29	0	5	4.25	mg/l
6	Total Hardness	941	13	7.17	5.5	26.2	0.08	°f
7	Nitrate	941	26	113.79	1.3	50.2	-0.05	mg/l
8	Conductivity	1003	436	53482.13	238	1953	3.27	microS/cm
9	Ammonium	942	0.04	0.0003	0	0.17	0.09	mg/l
10	Lead	23	1	1.33	1	5	2.93	mg/l
11	Sulfate	358	35	158.94	11	71	0.46	mg/l
12	Nickel	23	3	2.33	2	8	1.43	mg/l
13	Chloride	371	40	310.91	0.5	109	0.59	mg/l
14	Copper	23	0.05	0.047	0.02	0.74	1.24	mg/l
15	Aluminium	23	10	49.54	0.02	21	-0.09	mg/l
16	Antimony	23	0.5	0	0.5	0.5		mg/l
17	Atrazine	132	0.02	0.0005	0	0.13	1.21	mg/l
18	Benzo(1,12)perylene	23	0.003	3.32e-07	0.003	0.005	2.93	mg/l
19	Benzo(1,12)fluoranthen	23	0.003	3.32e-07	0.003	0.005	2.93	mg/l
20	Benzo(3,4)fluoranthene	23	0.003	3.32e-07	0.003	0.005	2.93	mg/l
21	Benzo(3,4)pyrene	23	0.003	4.74e-07	0.003	0.005	2.19	mg/l
22	Cadmium	23	0.05	0.04	0.1	0.5	-0.64	mg/l
23	Colour	939	5	5.67	0	7.5	-0.62	mg/l
24	Chrome	23	5	0	5	5		mg/l

25	Vinyl chloride	13	0.5	0	0.5	0.5		mg/l
26	Chlorites	16	0.015	14.03	0.015	15	3.61	mg/l
27	Total organic carbon	940	1.7	0.23	0.3	4.3	0.66	mg/l
28	thermotolerant coliform	357	0	0.15	0	7	16.67	/100ml
29	Faecal streptococci	366	0	0.005	0	1	13.42	/100ml
30	Aerobicmicrorg,rev37°c	366	0	1021.43	0	301	7.49	/ml
31	Aerobicmicrorg,rev22°c	366	0	1911.11	0	301	5.56	/ml
32	Dichloromonobromomethane	23	10.6	14.38	4.9	20.9	0.49	mg/l
33	Fluoranthene	23	0.003	2.44e-06	0.003	0.01	3.32	mg/l
34	Indopyrene	23	0.003	3.32e-07	0.003	0.005	2.93	mg/l
35	Monochlorodibromomethane	23	14.6	9.09	8.4	19	-0.14	mg/l
36	Nitrite	941	0.02	0.00009	0	0.03	-0.49	mg/l
37	Sum of 4 HPA	13	0.012	0	0.012	0.012		mg/l
38	Sum of T, H, M,	23	41.9	99.45	24.4	67	0.65	mg/l
39	Total alkali	941	1	0.23	0	1	-0.46	°f
40	Total alkali complete	941	6.7	2.63	0	16.2	0.33	°f
41	Tribromomethane	23	5.3	11.63	2	16.7	1.25	mg/l
42	Trichloromethane	23	6.9	25.15	1.7	24.5	1.59	mg/l
43	Turbidity	939	0.5	0.03	0.1	1.3	0.10	NTU

Table 2 Values of physical chemical parameters responsible for legionella positive and negative

Parameters	*Legionella spp* from water samples							
	Legionella positive Samples				Legionella negative Samples			
	Obs.	Mean	[95% CI]		Obs.	Mean	[95% CI]	
Free Chlorine	82	0.51	0.38	0.64	194	0.22	0.17	0.27
Water Temp.	83	20.15	18.79	21.52	199	16.74	15.68	17.81
pH	70	8.27	8.17	8.38	181	7.99	7.93	8.05
Total Hardness	81	14.11	13.49	14.73	190	13.84	13.49	14.18
Iron (Fe)	82	36.04	29.03	43.05	190	34.22	30.09	38.35
Nitrate	49	25.67	21.89	29.44	124	27.12	25.23	29.02
Conductivity	77	796.5	686.70	907.19	152	592.74	540.01	645.47

Table 3 Logistic regression of legionella and physical chemical parameters

Variables	**OR crude**	**95% CI**		**P-value**	**OR adj.**	**95% CI**		**P-value adj.**
Free Chlorine	3.38	1.93	5.91	<0.001	*			
pH	5.05	2.55	10.03	<0.001	*			
Water temperature	1.06	1.03	1.10	0.001	*			
Total Hardness	1.04	0.94	1.16	0.417	0.96	0.82	1.11	0.560
Nitrate	0.99	0.96	1.02	0.449	0.98	0.96	1.02	0.498
Iron (Fe)	1.002	0.99	1.01	0.645	1.003	0.99	1.01	0.595
Conductivity	1.001	1.001	1.002	0.000	*			

***P-value = <0.001**