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Persistent contamination of a hospital hot water network by *Legionella pneumophila*

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ABSTRACT

Objectives: We assessed the contamination with *Legionella pneumophila* (*Lp*) of the hot water network (HWN) of a hospital, mapped the risk of contamination, and evaluated the relatedness of isolates. We further validated phenotypically the biological features that could account for the contamination of the network.
Methods: We collected 360 water samples from October 2017 to September 2018 in 36 sampling points of a HWN of a building from a hospital in France. *Lp* were quantified and identified with culture-based methods and serotyping. *Lp* concentrations were correlated with water temperature, date and location of isolation. *Lp* isolates were genotyped by pulsed-field gel electrophoresis and compared to a collection of isolates retrieved in the same HWN two years later, or in other HWN from the same hospital.
Results: 207/360 (57.5%) samples were positive with *Lp*. In the hot water production system, *Lp* concentration was negatively associated with water temperature. In the distribution system, the risk of recovering *Lp* decreased when temperature was >55 °C ($p < 10^{-3}$), the proportion of samples with *Lp* increased with distance from the production network ($p < 10^{-3}$), and the risk of finding high loads of *Lp* increased 7.96 times in summer ($p = 0.001$). All *Lp* isolates (n = 135) were of serotype 3, and 134 (99.3%) shared the same pulsotype which is found two years later (Lp G). *In vitro* competition experiments showed that a 3-day culture of Lp G on agar inhibited the growth of a different pulsotype of *Lp* (Lp O) contaminating another HWN of the same hospital ($p = 0.050$). We also found that only Lp G survived to a 24h-incubation in water at 55 °C ($p = 0.014$).
Conclusion: We report here a persistent contamination with *Lp* of a hospital HWN. *Lp* concentrations were correlated with water temperature, season, and distance from the production system. Such persistent contamination could be due to biotic parameters such as intra-*Legionella* inhibition and tolerance to high temperature, but also to the non-optimal configuration of the HWN that prevented the maintenance of high temperature and optimal water circulation.

1. Introduction

Legionellae are bacteria found in natural aquatic and soil habitats. They are ubiquitous in many types of water sources and also contaminate man-made water systems, such as sanitary hospital hot-water networks (Emmerson, 2001). *Legionellae* are also opportunistic pathogens responsible for Legionnaire's disease (LD), a severe and life-threatening pneumonia, especially in patients with impaired host defenses (Miyashita et al., 2020). Humans are contaminated through the inhalation or aspiration of water aerosols containing *Legionellae*. In Europe, *Legionella pneumophila* (*Lp*) serogroup 1 is involved in the vast majority of LD cases (Campese et al., 2011; European Centre for Disease Prevention and Control, 2020). Most LD cases are community-acquired but LD is also a significant cause of hospital-acquired pneumonia (European Centre for Disease Prevention and Control, 2020; Lin et al., 2011). Although the number of nosocomial cases is low, the mortality rate of hospital-acquired LD is higher than community-acquired LD (Vincenti et al., 2019).

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Lp frequently colonizes the hot water networks (HWNs) of healthcare facilities, making it mandatory to monitor water colonization to reduce the risk of patient contamination (Arvand et al., 2011; Bargellini et al., 2011; Bédard et al., 2016; Martinelli et al., 2000; Serrano-Suárez et al., 2013; Stout et al., 2007). Although no infectious dose has yet been established, European countries have defined alert (100-1000 CFU/L) and action thresholds (1000–10,000 CFU/L) that trigger corrective measures (Bédard et al., 2016; Legifrance, 2010).

Legionellae contamination of hot water systems correlates with low water temperatures (Bédard et al., 2015, 2016; Dennis, 1990; Gavaldà et al., 2019). Hence, the prevention of *Legionellae* contamination in healthcare facilities relies, among other measures, on the maintenance of an elevated water temperature(Centre scientifique et technique du bâtiment, 2012). At the University Hospital of Besançon (UHB, France), *Lp* has contaminated the HWN of two buildings for more than 15 years. Persistent contamination by *Lp* has already been described in healthcare facilities due to the complexity and ageing of HWNs, which limit the efficiency of decontamination measures (Oberdorfer et al., 2008; Pancer et al., 2013; Perola et al., 2005).

However, the comprehensive and long-term mapping of the *Lp* contamination of a HWN and the genotypic and phenotypic characterization of the clone have rarely been carried out. Here, we assessed the *Lp* contamination of the UHB hot water system, mapped the risk of patient contamination, and evaluated the relatedness of the isolates that contaminated the different buildings. We further identified the risk factors that could have favored contamination of the UHB HWN by *Lp* and phenotypically validated the biological features (resistance to interbacterial competition and high temperatures) that could account for the long-term contamination of the network.

2. Materials and methods

Study setting. At the 1400-bed UHB, we sampled the HWNs of the grey and orange buildings, built in 1982 and 1999, respectively. Annual controls showed the grey building to be heavily and regularly contaminated by *Lp*. In the grey building, three stations produce hot water: the GB-NEA (Grey Building Northeast Area) production station, the GB-SWA (Grey Building Southwest Area) production station, and the GB-Basement production station. The GB-NEA consists of a semi-instantaneous looped system, which supplies patient rooms through two distribution networks (GB-EA, Grey Building East Area, and GB-NA, Grey Building North Area, Table 1). The GB-EA distribution network is composed of 17 distribution columns, 11 of which (GE01 to GE11) serve patient rooms on the second, third, and fourth floors (Table 1). Renovation work in the GB-EA distribution network led us to pay particular attention to its contamination by *Lp*. In the orange building, the HWN is a non-looped system and consists of a single semi-instantaneous hot water production station (Table 1).

Water sampling and temperature monitoring. For the GB-NEA HWN, we collected 1-L water samples monthly over one year, from October 2017 to September 2018, from 36 sampling points: three in the GB-NEA production network and 33 in the GB-EA distribution network. Water from the GB-NEA production network was sampled from the bottom of the water storage cylinder and from the loop departure and return points (n = 33). For the GB-EA distribution network, we drew 327 samples from the 11 columns serving the patient rooms on the three floors. Water samples were taken from points of use in patient rooms, such as showers or faucets, and in common showers when the latter were present and used in the care units. In total, we collected 360 samples from the GB-NEA water network and included five annual control samples in 2018 and 2020 for the NA distribution network (Table 1). Water samples from the orange building and other HWNs of the grey building (GB-SWA and GB-Basement) were also included in this study (n = 21) and were part of the annual control in 2018 and 2020 (Table 1).

Water was sampled according to French guidelines (*i.e.* after faucet accessory removal and a water pre-flush until the temperature has stabilized and within 2–3 min) ("PR FD T90-522", 2006). The maximum water temperature (T_{max}) was measured. The target temperature was >60 °C at the loop departure and >50 °C for all other points of the distribution network (Centre scientifique et technique du bâtiment, 2012).

Microbiological detection of *Legionellae*. We detected and quantified *Legionellae* and *Lp* according to French technical guidelines ("NF T90-431", 2017). Briefly, water was analyzed within 24 h after sampling. Two hundred microliters was plated on GVPC agar (Thermo Fisher Oxoid, Dardilly, France). In addition, 10 ml and 100 ml were filtered through 0.45-μm membranes (Merck, Darmstadt, Germany), treated 5 min with a pH 2.0 KCl–HCl buffer, placed on GVPC agar, and incubated at 36 °C for 8 to 11 days. We subcultured colonies for which the aspect was suggestive of *Legionellae* on BCYE agar, with or without L-cysteine (Thermofisher Oxoid, Dardilly, France). Colonies growing only on BCYE supplemented with L-cysteine agar were confirmed to be *Legionellae*, among which *Lp* was identified and serotyped by latex agglutination (Thermofisher Oxoid, Dardilly, France). We stored each *Lp* isolate in brain heart infusion broth supplemented with 30% glycerol at −80 °C until further analysis in the Centre de Ressources Biologiques-Filière Microbiologique of Besançon (Biobank number BB-0033-00090).

Genotyping. The clonal relatedness of *Lp* isolates was investigated by pulsed-field gel electrophoresis (PFGE) following *Sfi*I digestion, as

Table 1
Description of the hot water networks of the grey and orange buildings of the University Hospital of Besançon (France) and localization of the water samples.

Building	Hot water production network			Hot water distribution network (distribution columns)			
	Denomination	Collected samples (n)	Temperature (°C) (2018 and 2020)	Denomination	Floors supplied by distribution columns	Collected samples (n)	Temperature (°C) (2018 and 2020)
Grey building (GB)	Northeast Area (GB-NEA)	33	52.1 and 47.2	East Area (GB-EA)[a]	+1 to + 4	327	57.5 and 49.8
				North Area (GB-NA)[b]	+1 to + 8	5	NA and 48.2
	Southwest Area (GB-SWA)	2	NA and 52.2	West Area (GB-WA)[a]	+1 to + 8	1	NA and 48.2
				South Area (GB-SA)[b]	+1 to + 8	1	NA and 43.9
	Basement (GB-Basement)	3	53.4 and 51.0	Basement distribution	- 3 to 0	0	/
Orange building	Orange production	0	/	Orange distribution	- 2 to + 2	14	54.9 and 58.8

NA = Not Available.
[a] The GB-EA and GB-WA distribution networks are each composed of 17 distribution columns that supplied four and eight floors respectively.
[b] The GB-NA and GB-SA distribution networks are each composed of 16 distribution columns that both supplied eight floors.

previously described (Oberdorfer et al., 2008). We compared the restriction profiles using GelCompar software (Applied Maths, Kortrijk, Belgium) and defined pulsotypes and clusters according to international recommendations (Tenover et al., 1995).

Inter-*Legionella* inhibition assays on agar plates. We tested the inhibition between *Legionellae* isolates using competitive assays between a 'resident *Legionellae*' and a 'challenger *Legionellae*' as previously described (Fig. 3A) (Levin et al., 2019). We streaked 10^6 CFU of each tested isolate on BCYE plates containing 0.2 g/L of cysteine followed by incubation at 37 °C for 72 h (hereafter called 'resident *Legionellae*'). Then, 3-μL spots of a 10^8 CFU/ml suspension of *Legionellae* were plated 1 cm (near spot) and 2 cm (far spot) from the streak (hereafter called 'challenger *Legionellae*'). Once the spots were dry, the plates were incubated for an additional 72 h before scoring for inhibition. We quantified living bacteria by taking agar plugs from around each spot. The plugs were transferred into a saline solution, vortexed, and plated to quantify the CFUs per spot. The inhibitory power of each 'resident isolates' over the 'challenger isolates' was calculated as the ratio of CFUs in the far spots to that in the near spots. We tested the inhibition between the dominant pulsotypes of *Lp* that contaminated the water systems of the hospital orange building (Lp O) and grey building (Lp G) and used the strains *Lp* ATCC33152 and *L. anisa* (*La*) ATCC35292 as controls. All experiments were performed in triplicate with one isolate per pulsotype (*i.e.* Lp G and Lp O). Lp G representative isolate came from water sample collected in the GB-EA distribution network (at point of use of the third floor on the distribution column 05) in July 2018. Lp O representative isolate came from water sample collected in orange building distribution network during the annual control in July 2017.

Temperature-dependent survival assays. Local pulsotypes Lp O and Lp G and the control strains, *Lp* ATCC33152 and *La* ATCC35292, were suspended at a concentration of ~10^8 CFU/ml in tap water previously sterilized by 0.22-μm filtration. Suspensions were incubated at 50 °C, 55 °C, and 60 °C, which were the targeted temperatures of the HWN. The concentrations of the bacterial suspensions were determined at inoculation and after 24 h of incubation by plating on standard BCYE agar with a Spiral plater (Interscience, Saint-Nom la Bretèche). All experiments were performed in triplicate.

Statistical analysis. Stata software (version 14.1, Texas, USA) was used for statistical analysis. We tested the differences in the percentage of positive samples and the percentage of samples with *Lp* concentration $> 10^3$ CFU/L for the sampling points in the GB-NEA and GB-EA distribution networks using Fisher and $\chi 2$ tests. For the GB-NEA production network, *Lp* concentrations at the bottom of the storage water cylinder and the loop departure and return points were compared using the Kruskal-Wallis test. The correlations between the *Lp* concentrations and T_{max} measured for the three sampling points of the GB-NEA production network were studied using Spearman's coefficient. Multivariate analysis was used to evaluate the role of the location (floors and columns), temperature (<55 °C and ≥55 °C), and seasonality (autumn, winter, spring, and summer) for the EA distribution columns. Multivariate analysis using logistic regression models to estimate the adjusted odds ratios and 95% confidence intervals (95% CI) was performed using two variables: *Lp* positive results (presence or absence) and concentrations ($<10^3$ CFU/L or $> 10^3$ CFU/L).

We compared the inhibition ratios for the inhibition assays between *Legionellae* using the Kruskal-Wallis test coupled with a *post-hoc* Dunn test. These tests were also used to compare the proportion of bacteria surviving after incubation at various temperatures. Concentrations below the limit of detection were set to the limit of detection (*i.e.,* 1 CFU/ml) for statistical analysis. The α value was set to 0.05 for all tests.

3. Results and discussion

***L. pneumophila* serogroup 3 contaminates the GB-NEA hot water network.** We collected 360 samples from October 2017 to September 2018. Among them, 207 (57.5%) were positive for *Lp*: 28 (84.8%) for the water production network and 179 (54.7%) for the distribution network (Table 2). All sampling points were positive at least once during the sampling period.

The proportion of positive samples in the GB-NEA production network differed between the three sampling points ($p = 0.041$), with a higher percentage at the bottom of the water storage cylinder (100%) than in the loop departure (63.6%, $p = 0.037$, Table 2). The percentage of samples with an *Lp* concentration $> 10^3$ UFC/L also differed between the three sampling points ($p = 0.003$) and was lowest at the loop departure ($p = 0.003$, Table 2). In the distribution columns, the percentage of positive samples ranged from 43.0% (on the second floor) to 77.0% (on the fourth floor), where it was the highest ($p < 0.001$, Table 2). The percentage of samples with an *Lp* concentration $> 10^3$ UFC/L was lowest on the second floor ($p = 0.033$, Table 2). All isolates belonged to serogroup 3.

The L. *pneumophila* concentration correlates with water temperature. In the GB-NEA production network, the median *Lp* concentration ranged from 130 CFU/L (loop departure) to 37,500 CFU/L (bottom of the water storage cylinder). The median concentration was 5450 CFU/L and, as already mentioned, was highest at the bottom of the water storage cylinder ($p = 0.002$, Table 2). The targeted temperatures (*i.e.,* > 50 °C and >60 °C) were reached for two months during the study period for the loop departure, four months for the bottom of the water storage cylinder, and eight months for the loop return (Fig. 1). For the production sampling points, the *Lp* concentration was negatively associated with the T_{max} (Fig. 1). Hence, the highest concentration observed in March for the loop departure (35,000 CFU/L) and at the bottom of the water storage cylinder (750,000 CFU/L) were concomitant with the lowest temperatures, 55.9 °C and 27.4 °C, respectively (Fig. 1A and C). Similarly, there was a negative correlation for the loop return ($p = 0.009$), which was highly colonized from January to April (from 5600 CFU/L in March to 55,000 CFU/L in January), with T_{max} below or equal to the threshold (from 48.0 to 50.2 °C, Fig. 1B).

Minimizing *Lp* contamination in HWNs can be achieved by maintaining elevated water temperatures. Hence, many studies have reported a negative correlation between *Lp* concentration and temperature (Bédard et al., 2015, 2016; Gavaldà et al., 2019; Groothuis et al., 1985; Rhoads et al., 2015).

In the GB-EA distribution network, the median *Lp* concentration ranged from 0 CFU/L (second and third floors) to 175 UFC/L (fourth floor) (Table 2), with the concentration being highest on the fourth floor ($p = 0.001$, Table 2). The results of multivariate analysis indicated that the risk of recovering *Lp* was 6.49 times higher on this floor (95 CI = 2.89–14.58, Table 3). Both the third ($p = 0.008$) and fourth floors ($p = 0.045$, Table 3) were associated with *Lp* concentrations $> 10^3$ CFU/L. The risk of recovering *Lp* from the GB-EA distribution network decreased when the temperature was >55 °C ($p < 10^{-3}$, Table 3). Moreover, it has been shown that temperatures >55 °C in distribution networks more efficiently control *Legionellae* contamination than 50 °C (Gavaldà et al., 2019). Columns GE01 and GE02, which were the furthest away from the site of production, were also *Lp* positive ($p < 0.001$ for the two columns) and showed *Lp* concentrations $> 10^3$ CFU/L ($p < 0.001$ for the two columns, Table 3). This was presumably due to oversizing of the return sections of these two columns, leading to a decrease in circulation speed and stagnation of the water in the ring pipe. Hence, water circulation speed was lower than the minimum recommended 0.15 m s^{-1} (data not shown) (Centre scientifique et technique du bâtiment, 2012).

Concerning seasonality, the risk of *Lp* contamination and finding a concentration $> 10^3$ CFU/L was 2.90 times (95 CI = 1.27–6.63) and 4.50 times (95 CI = 1.60–12.64) higher in the spring, respectively (Table 3). Summer was associated with a higher risk of recovering an *Lp* concentration $> 10^3$ CFU/L (odds ratio = 7.96, 95 CI = 2.47–25.71) (Table 3). Such seasonal water system contamination by *Lp* has already been reported (Perrin et al., 2019).

The greater contamination of the GB-NEA with *Lp* was probably due to difficulty in reaching the targeted temperatures. This was particularly

Table 2
***Legionella pneumophila* contamination rates for the GB-NEA production and GB-EA distribution network of the University Hospital of Besançon from October 2017 to September 2018.**

Network location	Sampled site	Water samples collected (n)[1]	Positive samples (n, %)[2]	Samples with *Lp* concentration > 10^3 CFU/L (n, %)[3]	*Lp* median concentration (CFU/L)
GB-NEA production		**33**	**28 (84.8)**	**22 (66.7)**	**5,450**
	Bottom of the water storage cylinder	12	12 (100)	11 (91.7)	37,500
	Loop departure	11	7 (63.6)	3 (27.3)	130
	Loop return	10	9 (90.0)	8 (80.0)	4,200
GB-EA distribution		**327**	**179 (54.7)**	**81 (24.7)**	**10**
	Second floor	121	52 (43.0)	21 (17.4)	0^4
	Third floor	106	50 (47.2)	31 (29.3)	0^4
	Fourth floor	100	77 (77.0)	29 (29.0)	175
Total		**360**	**207 (57.5)**	**103 (28.6)**	**-**

[1] Number of sampling points sampled monthly for the GB-NEA production and distribution columns of the GB-EA distribution network.

[2] Number of positives samples with *L. pneumophila* among all water samples collected.

[3] Number of samples with a *L. pneumophila* concentration > 10^3 CFU/L among all water samples collected.

[4] Concentrations < 10 CFU/L were considered as 0 CFU/L for calculation.

Significant differences ($p < 0.05$) in the percentage of positive samples and the percentage of samples with a *Lp* concentration > 10^3 CFU/L according to the location in the GB-NEA production and GB-EA distribution networks were assessed using Fisher's Exact test.

Significant differences ($p < 0.05$) between *Lp* median concentrations were assessed using the Kruskal-Wallis test.

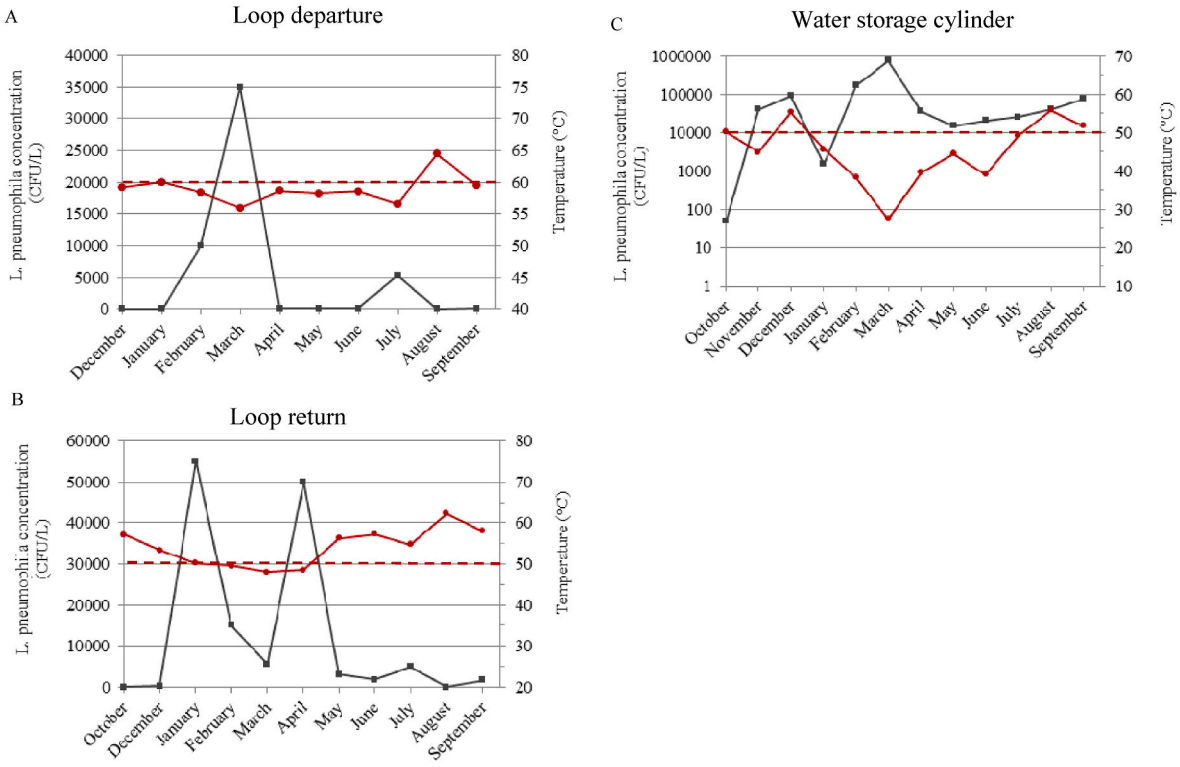

Fig. 1. Monthly concentration of *Legionella pneumophila* and maximum measured temperature (T_{max}) in the GB-NEA water production network of the University Hospital of Besançon from October 2017 to December 2018. (A) At the loop departure. (B) At the loop return. (C) At the bottom of water storage cylinder. Grey curves represent the monthly concentration of *Lp*, with a logarithmic scale for (C). Red curves represent the T_{max} measured during the sampling. Red dotted lines show the targeted temperature at the corresponding sampling points. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

true for the bottom of the water storage cylinder, which was always contaminated with a high load of *Lp* (Table 2). The difficulty in maintaining a continuously high temperature in this semi-instantaneous GB-NEA loop system may be due to an insufficiently heated water supply during periods of high water withdrawal (Centre scientifique et technique du bâtiment, 2012). The loop departure was less frequently contaminated with *Lp* than the bottom of the water storage cylinder (Table 2), although the targeted temperature (*i.e.,* > 60 °C) was rarely

Table 3
Logistic regression analysis for the association between the *Legionella* results and the location, maximum measured water temperature (T_{max}), and seasonality in the GB-EA distribution network of the University Hospital of Besançon.

	Positive L. *pneumophila* result			*L. pneumophila* concentration > 10^3 CFU/L		
	Adjusted OR[a]	95% CI[b]	*p*	Adjusted OR	95% CI	*P*
Floor						
Second floor	Reference	–	–	Reference	–	–
Third floor	1.68	0.81–3.48	0.17	2.86	1.32–6.20	0.008
Fourth floor	6.49	2.89–14.58	<0.001	2.28	1.02–5.12	0.045
Column						
GE03 to 11	Reference	–	–	Reference	–	–
GE02	21.68	5.66–83.08	<0.001	17.40	6.64–45.58	<0.001
GE01	32.80	6.80–158.26	<0.001	10.66	4.04–28.17	<0.001
T_{max}						
<55 °C	Reference	–	–	Reference	–	–
≥55 °C	0.033	0.012–0.088	<0.001	0.34	0.17–0.70	0.003
Seasonality						
Autumn	Reference	–	–	Reference	–	–
Winter	2.70	1.15–6.31	0.022	2.42	0.84–6.93	0.10
Spring	2.90	1.27–6.63	0.012	4.50	1.60–12.64	0.004
Summer	2.59	0.78–8.55	0.119	7.96	2.47–25.71	0.001

[a] OR, odds ratio.
[b] 95% CI, 95% confidence interval.

reached (Fig. 1A). However, temperatures were consistently >55 °C, contrary to the temperatures at the bottom of the water storage cylinder (Fig. 1C). Overall, the configuration of the hot water system prevented the maintenance of high temperatures and optimal water circulation, the two main preventive measures to reduce the risk of *Lp* contamination.

Specific pulsotypes persistently colonized the building hot water networks. We genotyped 187 isolates of *Lp* using PFGE, with 135 being retrieved from the GB-NEA HWN in 2017 and 2018. These were compared to isolates recovered from the GB-NEA in 2020 (n = 31) and from other HWNs of the UHB in 2018 and 2020 (GB-SWA network, n = 4; GB-Basement, n = 3; orange building HWN, n = 14). The vast majority of the isolates from the GB-NEA HWN (134 of 135 isolates) shared the same pulsotype, hereon called Lp G (Fig. 2). This pulsotypes was also shared by all isolates retrieved in 2018 and 2020 from other HWNs of the same building (*i.e.,* GB-SWA, GB-basement) and the GB-NEA two years later (in 2020) (Fig. 2).

In addition, most (12 out of 14) of the isolates cultured from the orange building clustered with another pulsotype (called Lp O) (Fig. 2). These data suggest that a single clone of *Lp* can persistently contaminate a HWN. Monoclonal contamination of HWNs with *Lp* has already been reported in six German hospitals (Oberdorfer et al., 2008). This suggests that co-colonization by multiple clones may be prevented by abiotic or biotic parameters, such as competition between subpopulations (Rangel-Frausto et al., 1999). Indeed, isolates with a type IV pilis and type II secretion systems would be able to colonize the biofilm and thus persist for a long time in water systems by competition with isolates without such systems (Lucas et al., 2006). Type IV pilis and type II secretion systems are very common amongst isolates; it is most likely that the *Lp* isolates tested here have genes coding for these systems.

Installed *Lp* isolates inhibit the growth of challenger *Lp*. The persistent contamination of a given HWN with a specific pulsotype of *Lp* led us to investigate how *Lp* isolates engage in inter-*Legionellae* competition. Using a previously described method (Levin et al., 2019), we found that the two pulsotypes resident in the grey and orange buildings (Lp G and Lp O, respectively) inhibited the growth of the challenger *Legionellae* plated 1 cm away on solid media (Fig. 3A). To quantify the observed inhibition, we recovered the challenger *Legionellae* grown at different distances from the resident *Legionellae* (Table S1). Although bacteria of the same pulsotype that were already growing on the plate slightly inhibited the challenger bacteria, the inhibition was not statistically significant (Fig. 3B, Table S1). On the contrary, we found a 16-fold difference in growth between Lp O antagonized by Lp G in the near spot *versus* Lp O plated outside of the zone of inhibition in the far spot (Fig. 3B). Similarly, Lp G growth was reduced 12-fold in the inhibition zone of Lp O (Kruskal-Wallis test, *p*-values = 0.050, followed by Dunn's test; Fig. 3B).

Interestingly, the observed inhibition did not target all *Legionellae*, as resident isolates of Lp G or Lp O did not inhibit the challengers *Lp* ATCC33152 and *La* ATCC35292 (Fig. 3B). Early studies suggested that *Lp* may compete with other *Legionellae* for similar biological niches (Wery et al., 2008) and it was recently proposed that established *Legionellae* communities may deploy molecules, such as homogentisic acid, that can protect against invasion by low-density competitors (Levin et al., 2019). Here, we validated that *Lp* installed in the grey building can inhibit the growth of *Lp* originating from the orange building, and *vice versa.*

Lp G and Lp O have a different tolerance to temperature. Environmental factors could be crucial for monoclonal contamination with *Lp* (David et al., 2017; Rodríguez-Martínez et al., 2015; Sharaby et al., 2017). Accordingly, water temperature influences the clonal diversity of *Legionellae,* which is lower in hot water than in cold water (Lesnik et al., 2016). Moreover, Sharaby et al. showed that different sites along a water network were dominated by three genotypes and that their location depended on the water temperature; these genotypes could behave as ecotypes, with distinct temperature ranges (Sharaby et al., 2017). As in other hospital buildings, the UHB HWN is composed of several ecological niches with different water temperatures, each favoring the implantation of a specific clone. Indeed, the median T_{max} in the HWN of the orange building varied from 55 °C to 60 °C between 2018 and 2020, whereas it varied from 44 °C to 57 °C between 2018 and 2020 in the hot water of the grey building (Table S3). We thus tested the ability of the Lp O and Lp G pulsotypes to survive in filter-sterilized sterile tap water at 50 °C, 55 °C, and 60 °C using *Lp* ATCC33152 and *La* ATCC35292 as controls. No strains remained culturable after 24h incubation at 60 °C (Fig. 4, Table S2). The proportion of *Legionellae* strains surviving at 50 °C varied from 4.13×10^{-5} to 2.46×10^{-4}, with no difference in the survival of Lp O and Lp G. At that temperature, we only found that Lp O was more persistent than *Lp* ATCC33152 (Kruskal-Wallis test, *p*-value = 0.016). This contrasted with the results for incubation at 55 °C for 24 h, after which Lp G persisted better than Lp O and *La* ATCC35292 (Kruskal-Wallis test, p-values = 0.014, Fig. 4, Table S2). Overall, we found that Lp G survived at a more stressful temperature (55 °C) than Lp O.

This result confirms that the resistance to high temperature varies between *Lp* clones (Sharaby et al., 2017). However, the incongruence between our results and the median T_{max} measured in the orange and

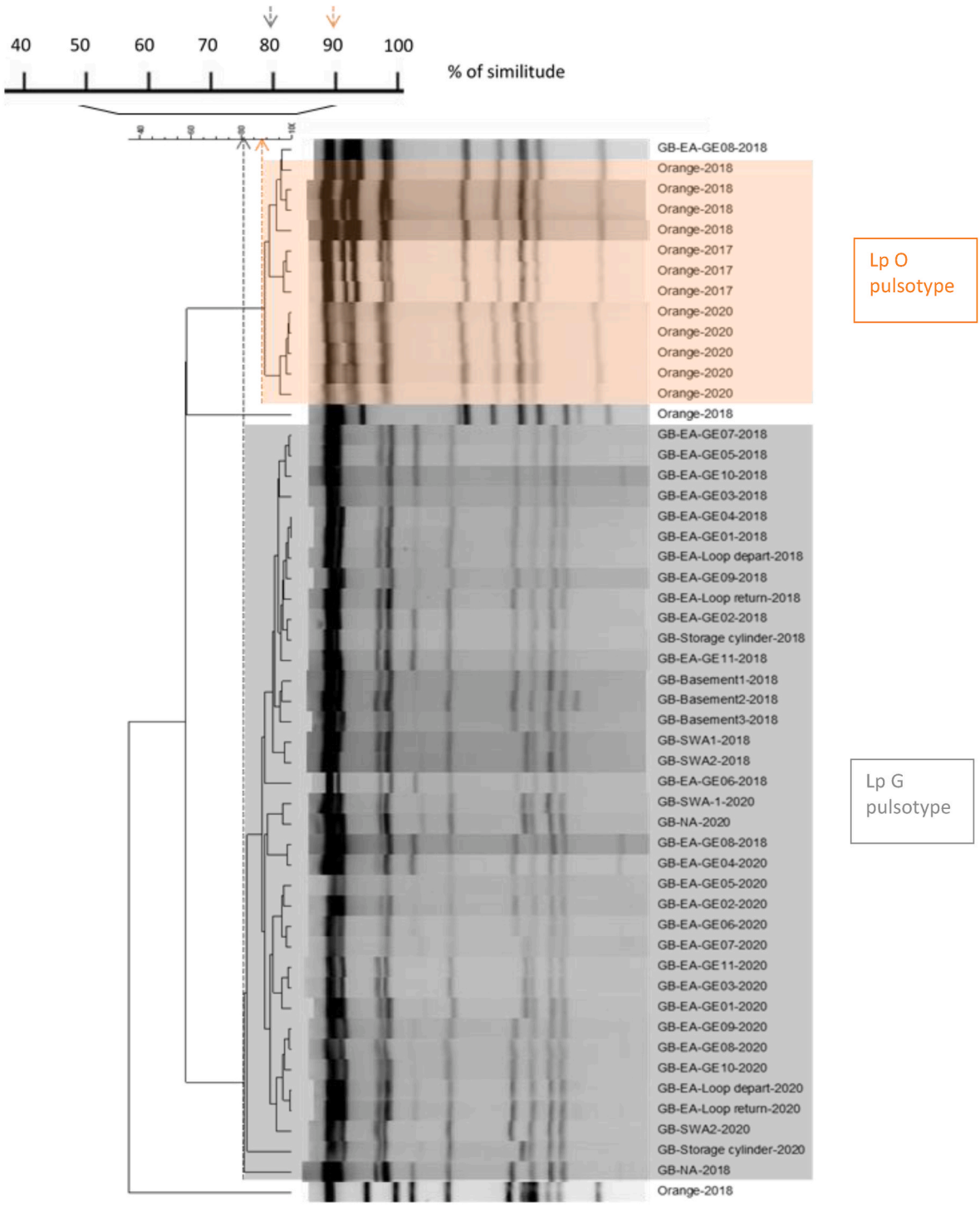

Fig. 2. Dendrogram representing the pulsotypes of *Legionella pneumophila* isolates from the grey and orange buildings of the University Hospital of Besançon between 2017 and 2020. For the GB-NEA HWN, 166 isolates were genotyped between 2018 and 2020 and among them, 165/166 shared the same genotype. For 2018 and 2020, one isolate per distribution column and per sampled site of the production network (water storage cylinder and loop departure and return points) were chosen to build the dendrogram. Thirty-one genotypes are represented and are named as follows: sample localization (GB-EA or GB-NA)-production or the number of the distribution column-year of collection. The genotypes of all isolates found at the sampled sites in 2017, 2018, and 2020 are represented in the dendrogram for the other HWNs of the grey (GB-SWA and GB-Basement) and orange buildings. Grey (Lp G pulsotype) and orange (Lp O pulsotype) boxes group the isolates from the HWNs of the orange and grey buildings, respectively, with the same pulsotype according to international recommendations. Orange and grey arrows show the percentage of similarity for the isolates from the orange and grey buildings, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

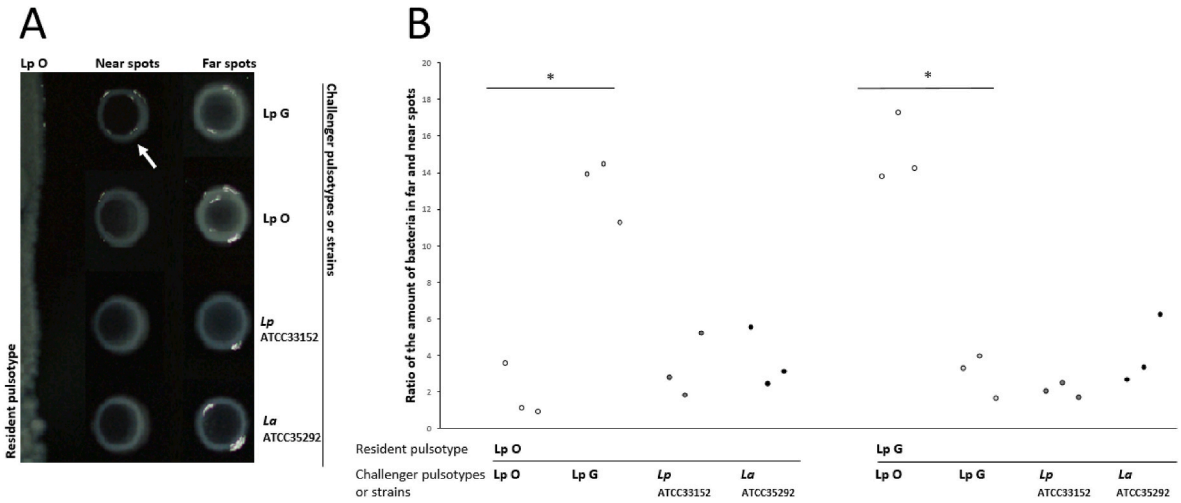

Fig. 3. *L. pneumophila* isolates of Lp G inhibit the growth of Lp O and *vice versa*. (A) When pre-incubated on low-cysteine BCYE charcoal agar plates, Lp O produces a zone of inhibition, affecting the growth of nearby *Legionellae*. Three-microliters of a 10^8 CFU/ml suspension of *Legionellae* were spotted in parallel columns three days after streaking Lp O. The white arrow indicates a zone of inhibition of the spot of Lp G 1 cm away the Lp O streak. (B) Quantification of the inhibition of challenger *Legionella* by the resident *Legionella*. Bacteria were removed from the plate in a plug of fixed area before plating for CFU counting. Data shown are the ratios of the CFU quantity in the far spots and that in the near spots. Each experiment was done in triplicate.

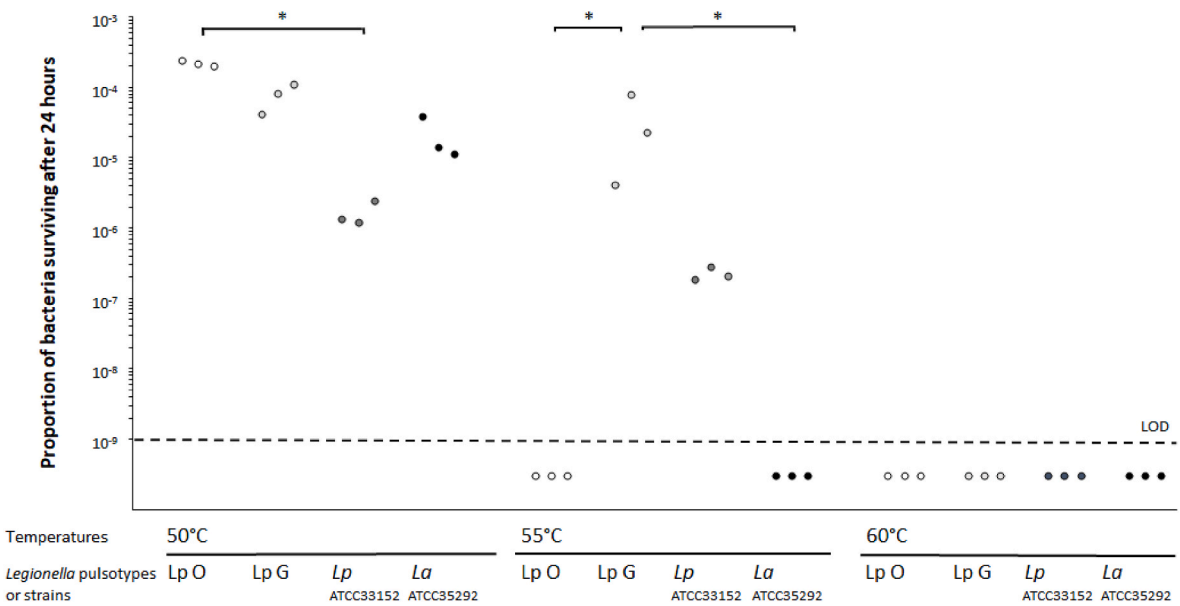

Fig. 4. The isolates of *Legionella pneumophila* Lp G and Lp O colonizing different buildings of the same hospital have different tolerance to temperature. We measured the ability to survive in filter-sterilized tap water at 50 °C, 55 °C, and 60 °C of Lp O (pulsotype colonizing the hot water system of orange building), Lp G (pulsotype colonizing the hot water system of grey building), and the reference strains *L. pneumophila* (*Lp*) ATCC33152 and *L. anisa* (*La*) ATCC35292. Each clone was suspended in water at 10^8 CFU/ml in triplicate and incubated 24 h. The results are shown as ratio of CFU/mL after 24 h incubation and to the CFU/mL before incubation. The limit of detection (LOD) was 1 CFU/ml and corresponded to a proportion of surviving bacteria of 1.00×10^{-9} (black dotted line).

grey buildings between 2018 and 2020 may be due to the evolution of Lp G in a system with drastic temperature changes. Hence, these conditions could have selected a pulsotype able to grow at low temperature and to survive in parts of the system with higher temperature. In the orange building, the more constant temperature will not allow this. Moreover, these results suggest also that other abiotic parameters may have also influenced the genotype distribution.

Limitations and strengths of the study. This study had several limitations. First, we only detailed the colonization by *Lp* in a portion of our hospital hot water system (the GB-NEA HWN). As sanitary controls of this portion had been strengthened due to hydraulic problems, this overestimated the percentage of samples positive for *Lp* for the whole hospital. Second, only one isolate of each positive sample was typed by PFGE, whereas it is known that up to three genetically different *Lp* genotypes can be found in one sample (Lück et al., 1998). Third, we explored the clonal relatedness of *Lp* isolates using PFGE. Despite its good performance and its wide use for epidemiological studies (Lück et al., 1998), this typing method is less discriminatory than genome-based typing methods (David et al., 2017). However, repeated sampling of all sites probably provided us with a complete picture of the genotypes contaminating the network. The long duration of the sampling period (2017–2020) is a strength of the study. We also paid particular attention to phenotypically explore the biological features that could account for the long-term association between a niche and a *Lp* clone.

Although no proven cases of nosocomial LD occurred during the

sampling period (2017–2020), corrective measures to control *Lp* contamination are needed. Measures implemented in the UHB (*i.e.,* thermal shocks, flushing of distribution columns, storage cylinder emptying) were insufficient to prevent long-term contamination of the HWN by *Lp*. These measures need be associated with optimization of the thermal and hydraulic performance of the HWN, such as the identification of dead ends and renovation of the networks themselves (Bédard et al., 2016).

4. Conclusion

We report persistent contamination of a hospital HWN with *Lp* serotype 3, with the distribution network being less contaminated than the production network. *Lp* concentrations were negatively associated with the T_{max} in the production network. We found that the risk of recovering *Lp* in the distribution network decreased when the temperature was >55 °C and increased in the spring and summer, as well as in the columns furthest away from the production network. The typing of isolates by PGFE showed each of the two sampled buildings to be persistently contaminated with a specific pulsotype of *Lp*. In-vitro experiments showed that *Lp* resident in one building inhibited the growth of the *Lp* originating from the other. Overall, we speculate that the suboptimal configuration of the HWN prevented the maintenance of sufficiently high temperatures and optimal water circulation, the two main preventive measures used to reduce the risk of *Lp* contamination.

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Author contributions

All authors contributed to the design or conduct of the study. DH, XB, and AJ developed the study protocol. SC, MR, and AJ collected the samples. SC, MR, AM, PC, DH, DH, and AJ performed or supervised the microbiological analyses. AM performed the statistical analyses. SC, AM, DH, MS, XB, and AJ drafted the manuscript and all authors reviewed and contributed to the manuscript. AJ coordinated the project.

Declaration of competing interest

None of the authors have anything to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114143.

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