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

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HIGHLIGHTS

- *Legionella pneumophila* (*Lp*) serogroup 3 contaminates a hospital hot water network
- Risk of recovering *Lp* increased with season and distance to hot water production
- Specific pulsotypes persistently colonized a hospital hot water network
- Resident *Lp* pulsotype in a hot water network inhibit the growth of challenger *Lp*
- Each resident *Lp* pulsotype has a different tolerance to temperature

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 $> 55^{\circ}\text{C}$ ($p < 10^{-3}$), 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

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

13 *Lp* frequently colonizes the hot water networks (HWNs) of healthcare facilities, making it
14 mandatory to monitor water colonization to reduce the risk of patient contamination (Arvand
15 et al., 2011; Bargellini et al., 2011; Bédard et al., 2016; Martinelli et al., 2000; Serrano-Suárez
16 et al., 2013; Stout et al., 2007). Although no infectious dose has yet been established,
17 European countries have defined alert (100-1,000 CFU/L) and action thresholds (1,000-
18 10,000 CFU/L) that trigger corrective measures (Bédard et al., 2016).

19 *Legionellae* contamination of hot water systems correlates with low water temperatures
20 (Bédard et al., 2016, 2015; Dennis, 1990; Gavaldà et al., 2019). Hence, the prevention of
21 *Legionellae* contamination in healthcare facilities relies, among other measures, on the
22 maintenance of an elevated water temperature (“Guide technique”, 2012). At the University
23 Hospital of Besançon (UHB, France), *Lp* has contaminated the HWN of two buildings for
24 more than 15 years. Persistent contamination by *Lp* has already been described in healthcare

25 facilities due to the complexity and ageing of HWNs, which limit the efficiency of
26 decontamination measures (Oberdorfer et al., 2008; Pancer et al., 2013; Perola et al., 2005).

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

35

36 MATERIALS AND METHODS

37 **Study setting.** At the 1,400-bed UHB, we sampled the HWNs of the grey and orange
38 buildings, built in 1982 and 1999, respectively. Annual controls showed the grey building to
39 be heavily and regularly contaminated by *Lp*. In the grey building, three stations produce hot
40 water: the GB-NEA (Grey Building Northeast Area) production station, the GB-SWA (Grey
41 Building Southwest Area) production station, and the GB-Basement production station. The
42 GB-NEA consists of a semi-instantaneous looped system, which supplies patient rooms
43 through two distribution networks (GB-EA, Grey Building East Area, and GB-NA, Grey
44 Building North Area, Table 1). The GB-EA distribution network is composed of 17
45 distribution columns, 11 of which (GE01 to GE11) serve patient rooms on the second, third,
46 and fourth floors (Table 1). Renovation work in the GB-EA distribution network led us to pay
47 particular attention to its contamination by *Lp*. In the orange building, the HWN is a non-
48 looped system and consists of a single semi-instantaneous hot water production station (Table
49 1).

50 **Water sampling and temperature monitoring.** For the GB-NEA HWN, we collected one-
51 liter water samples monthly over one year, from October 2017 to September 2018, from 36
52 sampling points: three in the GB-NEA production network and 33 in the GB-EA distribution
53 network. Water from the GB-NEA production network was sampled from the bottom of the
54 water storage cylinder and from the loop departure and return points ($n = 33$). For the GB-EA
55 distribution network, we drew 327 samples from the 11 columns serving the patient rooms on
56 the three floors. Water samples were taken from points of use in patient rooms, such as
57 showers or faucets, and in common showers when the latter were present and used in the care
58 units. In total, we collected 360 samples from the GB-NEA water network and included five
59 annual control samples in 2018 and 2020 for the NA distribution network (Table 1). Water

60 samples from the orange building and other HWNs of the grey building (GB-SWA and GB-
61 Basement) were also included in this study (n = 21) and were part of the annual control in
62 2018 and 2020 (Table 1).

63 Water was sampled according to French guidelines (*i.e.* after faucet accessory removal and-a
64 water pre-flush until the temperature has stabilized and within 2 to 3 minutes) (“PR FD T90-
65 522”, 2006). The maximum water temperature (T_{\max}) was measured. The target temperature
66 was $> 60^{\circ}\text{C}$ at the loop departure and $> 50^{\circ}\text{C}$ for all other points of the distribution network
67 (“Guide technique”, 2012).

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

81 **Genotyping.** The clonal relatedness of *Lp* isolates was investigated by pulsed-field gel
82 electrophoresis (PFGE) following *SfiI* digestion, as previously described (Oberdorfer et al.,
83 2008). We compared the restriction profiles using GelCompar software (Applied Maths,

84 Kortrijk, Belgium) and defined pulsotypes and clusters according to international
85 recommendations (Tenover et al., 1995).

86 **Inter-*Legionella* inhibition assays on agar plates.** We tested the inhibition between
87 *Legionellae* isolates using competitive assays between a ‘resident *Legionellae*’ and a
88 ‘challenger *Legionellae*’ as previously described (Figure 3A) (Levin et al., 2019). We
89 streaked 10^6 CFU of each tested isolate on BCYE plates containing 0.2 g/L of cysteine
90 followed by incubation at 37°C for 72 h (hereafter called ‘resident *Legionellae*’). Then, 3- μ L
91 spots of a 10^8 CFU/ml suspension of *Legionellae* were plated 1 cm (near spot) and 2 cm (far
92 spot) from the streak (hereafter called ‘challenger *Legionellae*’). Once the spots were dry, the
93 plates were incubated for an additional 72 h before scoring for inhibition. We quantified
94 living bacteria by taking agar plugs from around each spot. The plugs were transferred into a
95 saline solution, vortexed, and plated to quantify the CFUs per spot. The inhibitory power of
96 each ‘resident isolates’ over the ‘challenger isolates’ was calculated as the ratio of CFUs in
97 the far spots to that in the near spots. We tested the inhibition between the dominant
98 pulsotypes of *Lp* that contaminated the water systems of the hospital orange building (*Lp* O)
99 and grey building (*Lp* G) and used the strains *Lp* ATCC33152 and *L. anisa* (*La*) ATCC35292
100 as controls. All experiments were performed in triplicate with one isolate per pulsotype (*i.e.*
101 *Lp* G and *Lp* O). *Lp* G representative isolate came from water sample collected in the GB-EA
102 distribution network (at point of use of the third floor on the distribution column 05) in July
103 2018. *Lp* O representative isolate came from water sample collected in orange building
104 distribution network during the annual control in July 2017.

105 **Temperature-dependent survival assays.** Local pulsotypes *Lp* O and *Lp* G and the control
106 strains, *Lp* ATCC33152 and *La* ATCC35292, were suspended at a concentration of $\sim 10^8$
107 CFU/ml in tap water previously sterilized by 0.22- μ m filtration. Suspensions were incubated
108 at 50°C, 55°C, and 60°C, which were the targeted temperatures of the HWN. The
109 concentrations of the bacterial suspensions were determined at inoculation and after 24 h of

110 incubation by plating on standard BCYE agar with a Spiral plater (Interscience, Saint-Nom la
111 Bretèche). All experiments were performed in triplicate.

112 **Statistical analysis.** Stata software (version 14.1, Texas, USA) was used for statistical
113 analysis. We tested the differences in the percentage of positive samples and the percentage of
114 samples with *Lp* concentration $> 10^3$ CFU/L for the sampling points in the GB-NEA and GB-
115 EA distribution networks using Fisher and χ^2 tests. For the GB-NEA production network, *Lp*
116 concentrations at the bottom of the storage water cylinder and the loop departure and return
117 points were compared using the Kruskal-Wallis test. The correlations between the *Lp*
118 concentrations and T_{\max} measured for the three sampling points of the GB-NEA production
119 network were studied using Spearman's coefficient. Multivariate analysis was used to
120 evaluate the role of the location (floors and columns), temperature ($< 55^\circ\text{C}$ and $\geq 55^\circ\text{C}$), and
121 seasonality (autumn, winter, spring, and summer) for the EA distribution columns.
122 Multivariate analysis using logistic regression models to estimate the adjusted odds ratios and
123 95% confidence intervals (95% CI) was performed using two variables: *Lp* positive results
124 (presence or absence) and concentrations ($< 10^3$ CFU/L or $> 10^3$ CFU/L).

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

130

131 RESULTS AND DISCUSSION

132 ***L. pneumophila* serogroup 3 contaminates the GB-NEA hot water network.** We collected
133 360 samples from October 2017 to September 2018. Among them, 207 (57.5%) were positive
134 for *Lp*: 28 (84.8%) for the water production network and 179 (54.7%) for the distribution
135 network (Table 2). All sampling points were positive at least once during the sampling period.
136 The proportion of positive samples in the GB-NEA production network differed between the
137 three sampling points ($p = 0.041$), with a higher percentage at the bottom of the water storage
138 cylinder (100%) than in the loop departure (63.6%, $p = 0.037$, Table 2). The percentage of
139 samples with an *Lp* concentration $> 10^3$ UFC/L also differed between the three sampling
140 points ($p = 0.003$) and was lowest at the loop departure ($p = 0.003$, Table 2). In the
141 distribution columns, the percentage of positive samples ranged from 43.0% (on the second
142 floor) to 77.0% (on the fourth floor), where it was the highest ($p < 0.001$, Table 2). The
143 percentage of samples with an *Lp* concentration $> 10^3$ UFC/L was lowest on the second floor
144 ($p = 0.033$, Table 2). All isolates belonged to serogroup 3.

145 **The *L. pneumophila* concentration correlates with water temperature.** In the GB-NEA
146 production network, the median *Lp* concentration ranged from 130 CFU/L (loop departure) to
147 37,500 CFU/L (bottom of the water storage cylinder). The median concentration was 5,450
148 CFU/L and, as already mentioned, was highest at the bottom of the water storage cylinder (p
149 $= 0.002$, Table 2). The targeted temperatures (*i.e.*, $> 50^\circ\text{C}$ and $> 60^\circ\text{C}$) were reached for two
150 months during the study period for the loop departure, four months for the bottom of the
151 water storage cylinder, and eight months for the loop return (Figure 1). For the production
152 sampling points, the *Lp* concentration was negatively associated with the T_{max} (Figure 1).
153 Hence, the highest concentration observed in March for the loop departure (35,000 CFU/L)
154 and at the bottom of the water storage cylinder (750,000 CFU/L) were concomitant with the

155 lowest temperatures, 55.9°C and 27.4°C, respectively (Figures 1A and 1C). Similarly, there
156 was a negative correlation for the loop return ($p = 0.009$), which was highly colonized from
157 January to April (from 5,600 CFU/L in March to 55,000 CFU/L in January), with T_{\max} below
158 or equal to the threshold (from 48.0 to 50.2°C, Figure 1B).

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

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

178

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

184 The greater contamination of the GB-NEA with *Lp* was probably due to difficulty in reaching
185 the targeted temperatures. This was particularly true for the bottom of the water storage
186 cylinder, which was always contaminated with a high load of *Lp* (Table 2). The difficulty in
187 maintaining a continuously high temperature in this semi-instantaneous GB-NEA loop system
188 may be due to an insufficiently heated water supply during periods of high water withdrawal
189 (“Guide technique”, 2012). The loop departure was less frequently contaminated with *Lp* than
190 the bottom of the water storage cylinder (Table 2), although the targeted temperature (*i.e.*, $>$
191 60°C) was rarely reached (Figure 1A). However, temperatures were consistently $> 55^{\circ}\text{C}$,
192 contrary to the temperatures at the bottom of the water storage cylinder (Figure 1C). Overall,
193 the configuration of the hot water system prevented the maintenance of high temperatures and
194 optimal water circulation, the two main preventive measures to reduce the risk of *Lp*
195 contamination.

196 **Specific pulsotypes persistently colonized the building hot water networks.** We genotyped
197 187 isolates of *Lp* using PFGE, with 135 being retrieved from the GB-NEA HWN in 2017
198 and 2018. These were compared to isolates recovered from the GB-NEA in 2020 ($n = 31$) and
199 from other HWNs of the UHB in 2018 and 2020 (GB-SWA network, $n = 4$; GB-Basement, n
200 $= 3$; orange building HWN, $n = 14$). The vast majority of the isolates from the GB-NEA
201 HWN (134 of 135 isolates) shared the same pulsotype, hereon called *Lp* G (Figure 2). This
202 pulsotypes was also shared by all isolates retrieved in 2018 and 2020 from other HWNs of the

203 same building (*i.e.*, GB-SWA, GB-basement) and the GB-NEA two years later (in 2020)
204 (Figure 2).

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

215 **Installed *Lp* isolates inhibit the growth of challenger *Lp*.** The persistent contamination of a
216 given HWN with a specific pulsotype of *Lp* led us to investigate how *Lp* isolates engage in
217 inter-*Legionellae* competition. Using a previously described method (Levin et al. 2019), we
218 found that the two pulsotypes resident in the grey and orange buildings (Lp G and Lp O,
219 respectively) inhibited the growth of the challenger *Legionellae* plated 1 cm away on solid
220 media (Figure 3A). To quantify the observed inhibition, we recovered the challenger
221 *Legionellae* grown at different distances from the resident *Legionellae* (Table S2). Although
222 bacteria of the same pulsotype that were already growing on the plate slightly inhibited the
223 challenger bacteria, the inhibition was not statistically significant (Figure 3B, Table S1). On
224 the contrary, we found a 16-fold difference in growth between Lp O antagonized by Lp G in
225 the near spot *versus* Lp O plated outside of the zone of inhibition in the far spot (Figure 3B).

226 Similarly, Lp G growth was reduced 12-fold in the inhibition zone of Lp O (Kruskal-Wallis
227 test, p -values = 0.050, followed by Dunn's test; Figure 3B).

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

236 **Lp G and Lp O have a different tolerance to temperature.** Environmental factors could be
237 crucial for monoclonal contamination with *Lp* (David et al., 2017; Rodríguez-Martínez et al.,
238 2015; Sharaby et al., 2017). Accordingly, water temperature influences the clonal diversity of
239 *Legionellae*, which is lower in hot water than in cold water (Lesnik et al., 2016). Moreover,
240 Sharaby *et al.* showed that different sites along a water network were dominated by three
241 genotypes and that their location depended on the water temperature; these genotypes could
242 behave as ecotypes, with distinct temperature ranges (Sharaby et al., 2017). As in other
243 hospital buildings, the UHB HWN is composed of several ecological niches with different
244 water temperatures, each favoring the implantation of a specific clone. Indeed, the median
245 T_{\max} in the HWN of the orange building varied from 55°C to 60°C between 2018 and 2020,
246 whereas it varied from 44°C to 57°C between 2018 and 2020 in the hot water of the grey
247 building (Table S3). We thus tested the ability of the Lp O and Lp G pulsotypes to survive in
248 filter-sterilized sterile tap water at 50°C, 55°C, and 60°C using *Lp* ATCC33152 and *La*
249 ATCC35292 as controls. No strains remained culturable after 24h incubation at 60°C (Figure

250 4, Table S2). The proportion of *Legionellae* strains surviving at 50°C varied from 4.13×10^{-5}
251 to 2.46×10^{-4} , with no difference in the survival of Lp O and Lp G. At that temperature, we
252 only found that Lp O was more persistent than *Lp* ATCC33152 (Kruskal-Wallis test, p -value
253 = 0.016). This contrasted with the results for incubation at 55°C for 24 h, after which Lp G
254 persisted better than Lp O and *La* ATCC35292 (Kruskal-Wallis test, p -values = 0.014, Figure
255 4, Table S2). Overall, we found that Lp G survived at a more stressful temperature (55°C)
256 than Lp O.

257 This result confirms that the resistance to high temperature varies between *Lp* clones (Sharaby
258 et al., 2017). However, the incongruence between our results and the median T_{\max} measured
259 in the orange and grey buildings between 2018 and 2020 may be due to the evolution of Lp G
260 in a system with drastic temperature changes. Hence, these conditions could have selected a
261 pulsotype able to grow at low temperature and to survive in parts of the system with higher
262 temperature. In the orange building, the more constant temperature will not allow this.
263 Moreover, these results suggest also that other abiotic parameters may have also influenced
264 the genotype distribution.

265 **Limitations and strengths of the study.** This study had several limitations. First, we only
266 detailed the colonization by *Lp* in a portion of our hospital hot water system (the GB-NEA
267 HWN). As sanitary controls of this portion had been strengthened due to hydraulic problems,
268 this overestimated the percentage of samples positive for *Lp* for the whole hospital. Second,
269 only one isolate of each positive sample was typed by PFGE, whereas it is known that up to
270 three genetically different *Lp* genotypes can be found in one sample (Lück et al., 1998).
271 Third, we explored the clonal relatedness of *Lp* isolates using PFGE. Despite its good
272 performance and its wide use for epidemiological studies (Lück et al., 1998), this typing
273 method is less discriminatory than genome-based typing methods (David et al., 2017).

274 However, repeated sampling of all sites probably provided us with a complete picture of the
275 genotypes contaminating the network. The long duration of the sampling period (2017-2020)
276 is a strength of the study. We also paid particular attention to phenotypically explore the
277 biological features that could account for the long-term association between a niche and a *Lp*
278 clone.

279 Although no proven cases of nosocomial LD occurred during the sampling period (2017 to
280 2020), corrective measures to control *Lp* contamination are needed. Measures implemented in
281 the UHB (*i.e.*, thermal shocks, flushing of distribution columns, storage cylinder emptying)
282 were insufficient to prevent long-term contamination of the HWN by *Lp*. These measures
283 need be associated with optimization of the thermal and hydraulic performance of the HWN,
284 such as the identification of dead ends and renovation of the networks themselves (Bédard et
285 al., 2016).

286

287 **CONCLUSION**

288 We report persistent contamination of a hospital HWN with *Lp* serotype 3, with the
289 distribution network being less contaminated than the production network. *Lp* concentrations
290 were negatively associated with the T_{\max} in the production network. We found that the risk of
291 recovering *Lp* in the distribution network decreased when the temperature was $> 55^{\circ}\text{C}$ and
292 increased in the spring and summer, as well as in the columns furthest away from the
293 production network. The typing of isolates by PGFE showed each of the two sampled
294 buildings to be persistently contaminated with a specific pulsotype of *Lp*. In-vitro experiments
295 showed that *Lp* resident in one building inhibited the growth of the *Lp* originating from the
296 other. Overall, we speculate that the suboptimal configuration of the HWN prevented the

297 maintenance of sufficiently high temperatures and optimal water circulation, the two main
298 preventive measures used to reduce the risk of *Lp* contamination.
299

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302

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304

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307

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

313

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430
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432

433 **TABLE AND FIGURE CAPTIONS**

434 **Table 1. Description of the hot water networks of the grey and orange buildings of the**
435 **University Hospital of Besançon and localization of water samples.**

436

437 **Table 2. *Legionella pneumophila* contamination rates for the GB-NEA production and**
438 **GB-EA distribution networks of the grey building of the University Hospital of**
439 **Besançon (France) from October 2017 to September 2018.**

440

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

445

446 **Figure 1. Monthly concentration of *Legionella pneumophila* and maximum measured**
447 **temperature (T_{\max}) in the GB-NEA water production network of the University Hospital**
448 **of Besançon from October 2017 to December 2018. (A) At the loop departure. (B) At the**
449 **loop return. (C) At the bottom of water storage cylinder. Grey curves represent the monthly**
450 **concentration of *Lp*, with a logarithmic scale for (C). Red curves represent the T_{\max} measured**
451 **during the sampling. Red dotted lines show the targeted temperature at the corresponding**
452 **sampling points.**

453

454 **Figure 2. Dendrogram representing the pulsotypes of *Legionella pneumophila* isolates**
455 **from the grey and orange buildings of the University Hospital of Besançon between 2017**
456 **and 2020. For the GB-NEA HWN, 166 isolates were genotyped between 2018 and 2020 and**

457 among them, 165/166 shared the same genotype. For 2018 and 2020, one isolate per
458 distribution column and per sampled site of the production network (water storage cylinder
459 and loop departure and return points) were chosen to build the dendrogram. Thirty-one
460 genotypes are represented and are named as follows: sample localization (GB-EA or GB-
461 NA)-production or the number of the distribution column-year of collection. The genotypes of
462 all isolates found at the sampled sites in 2017, 2018, and 2020 are represented in the
463 dendrogram for the other HWNs of the grey (GB-SWA and GB-Basement) and orange
464 buildings. Grey (Lp G pulsotype) and orange (Lp O pulsotype) boxes group the isolates from
465 the HWNs of the orange and grey buildings, respectively, with the same pulsotype according
466 to international recommendations. Orange and grey arrows show the percentage of similarity
467 for the isolates from the orange and grey buildings, respectively.

468

469 **Figure 3. *L. pneumophila* isolates of Lp G inhibit the growth of Lp O and vice versa. (A)**

470 When pre-incubated on low-cysteine BCYE charcoal agar plates, Lp O produces a zone of
471 inhibition, affecting the growth of nearby *Legionellae*. Three-microliters of a 10^8 CFU/ml
472 suspension of *Legionellae* were spotted in parallel columns three days after streaking Lp O.
473 The white arrow indicates a zone of inhibition of the spot of Lp G 1 cm away the Lp O streak.

474 **(B) Quantification of the inhibition of challenger *Legionella* by the resident *Legionella*.**

475 Bacteria were removed from the plate in a plug of fixed area before plating for CFU counting.
476 Data shown are the ratios of the CFU quantity in the far spots and that in the near spots. Each
477 experiment was done in triplicate.

478

479 **Figure 4. The isolates of *Legionella pneumophila* Lp G and Lp O colonizing different**

480 **buildings of the same hospital have different tolerance to temperature.** We measured the

481 ability to survive in filter-sterilized tap water at 50°C, 55°C, and 60°C of Lp O (pulsotype

482 colonizing the hot water system of orange building), Lp G (pulsotype colonizing the hot water
483 system of grey building), and the reference strains *L. pneumophila* (*Lp*) ATCC33152 and
484 *L. anisa* (*La*) ATCC35292. Each clone was suspended in water at 10^8 CFU/ml in triplicate
485 and incubated 24 hours. The results are shown as ratio of CFU/mL after 24 hours incubation
486 and to the CFU/mL before incubation. The limit of detection (LOD) was 1 CFU/ml and
487 corresponded to a proportion of surviving bacteria of 1.00×10^{-9} (black dotted line).

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) ¹	+ 1 to + 4	327	57.5 and 49.8
				North Area (GB-NA) ²	+ 1 to + 8	5	NA and 48.2
	Southwest Area (GB-SWA)	2	NA and 52.2	West Area (GB-WA) ¹	+ 1 to + 8	1	NA and 48.2
				South Area (GB-SA) ²	+ 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

¹The GB-EA and GB-WA distribution networks are each composed of 17 distribution columns that supplied four and eight floors respectively.

²The GB-NA and GB-SA distribution networks are each composed of 16 distribution columns that both supplied eight floors.

NA = Not Available

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 <i>Lp</i> concentration > 10 ³ CFU/L (n, %) ³	<i>Lp</i> 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 > 10³ 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 > 10³ 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.

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 <i>L. pneumophila</i> result			<i>L. pneumophila</i> concentration > 10 ³ CFU/L		
	Adjusted OR ¹	95% CI ²	<i>p</i>	Adjusted OR	95% CI	<i>p</i>
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

¹ OR, odds ratio

² 95% CI, 95% confidence interval

Figure 1

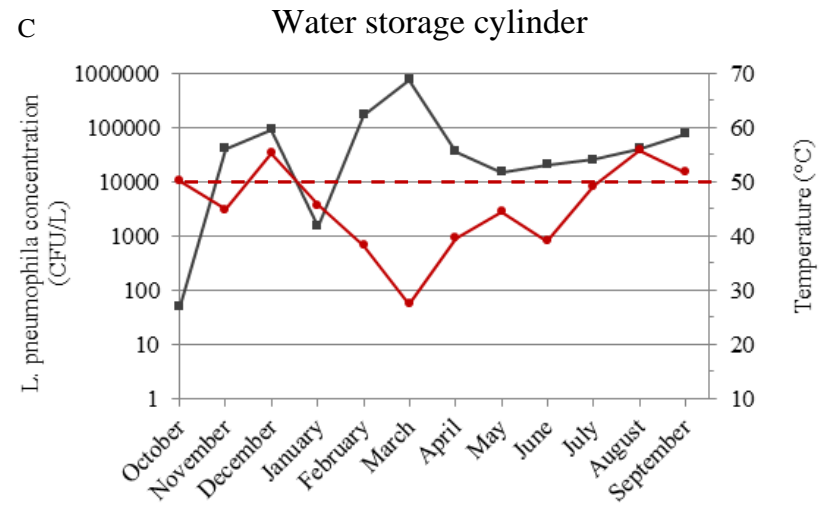
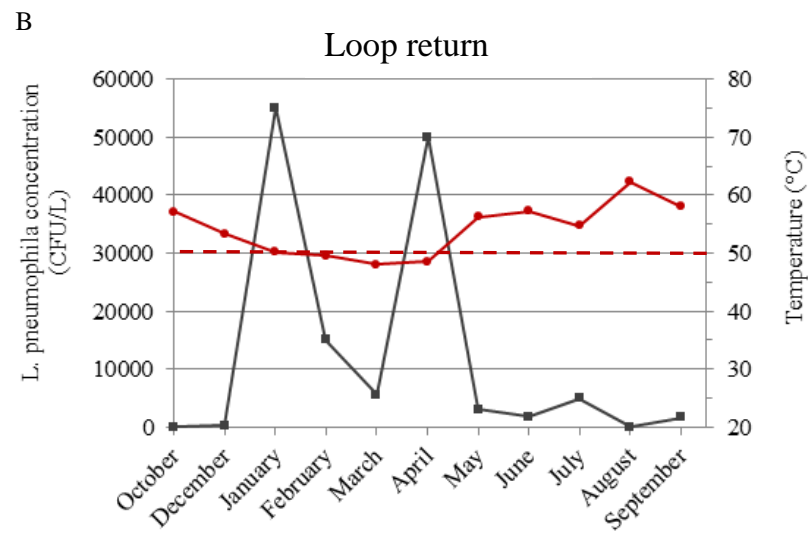
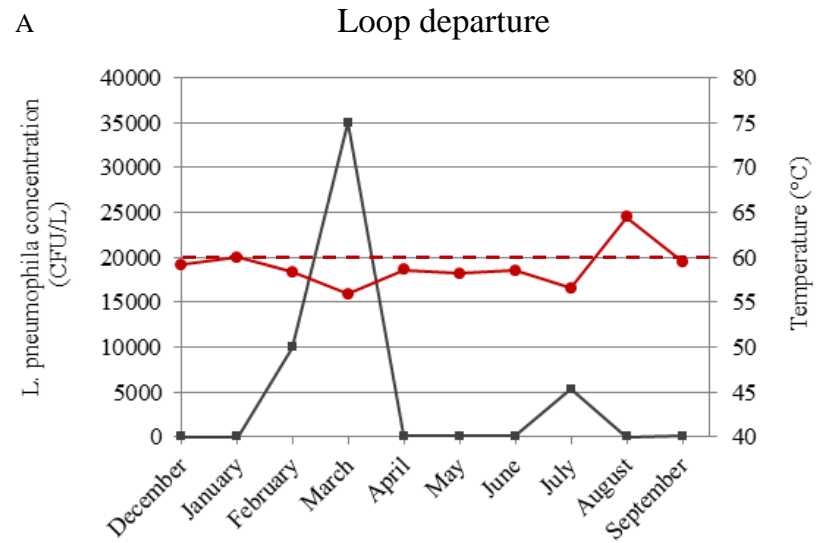


Figure 2

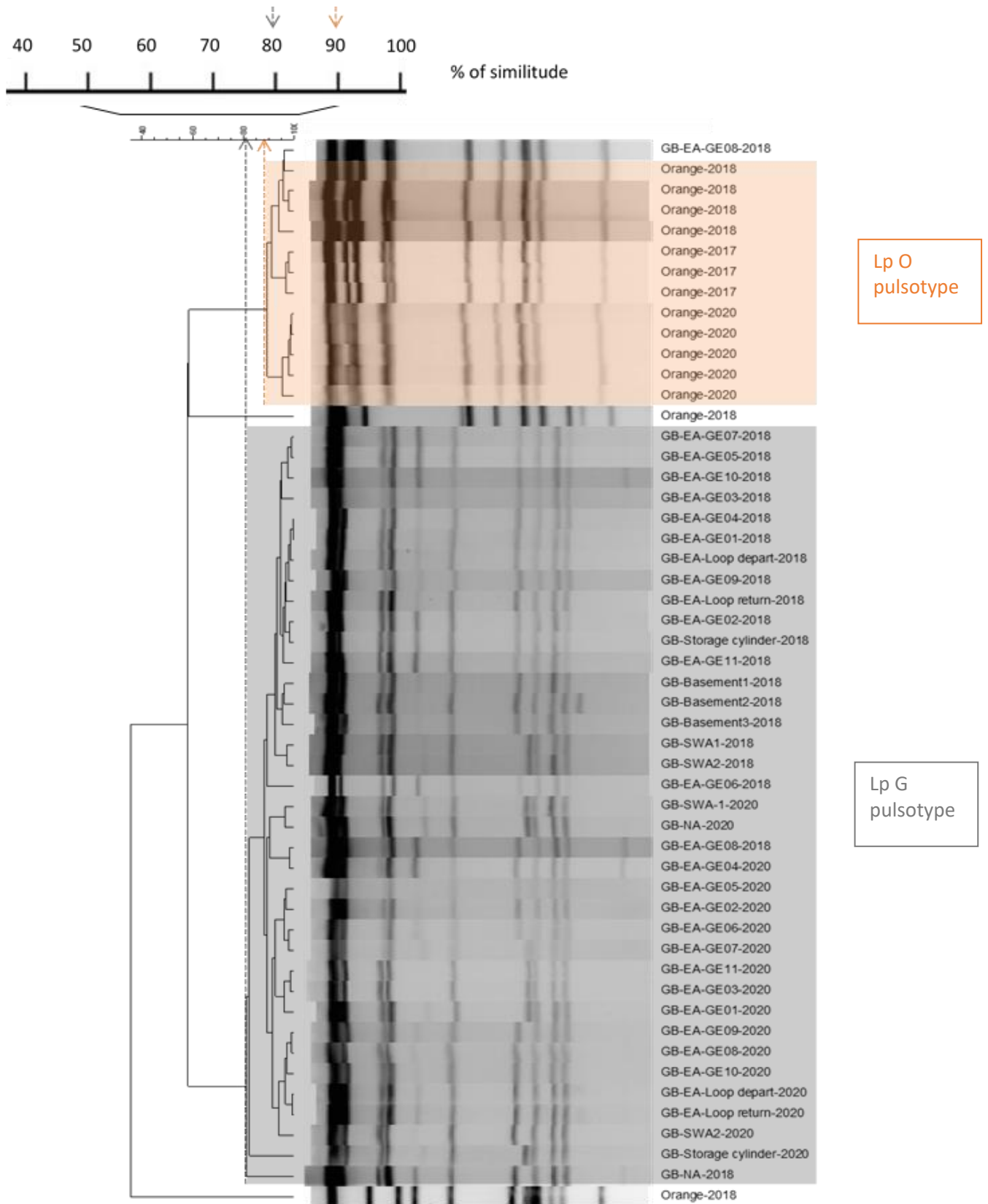


Figure 3

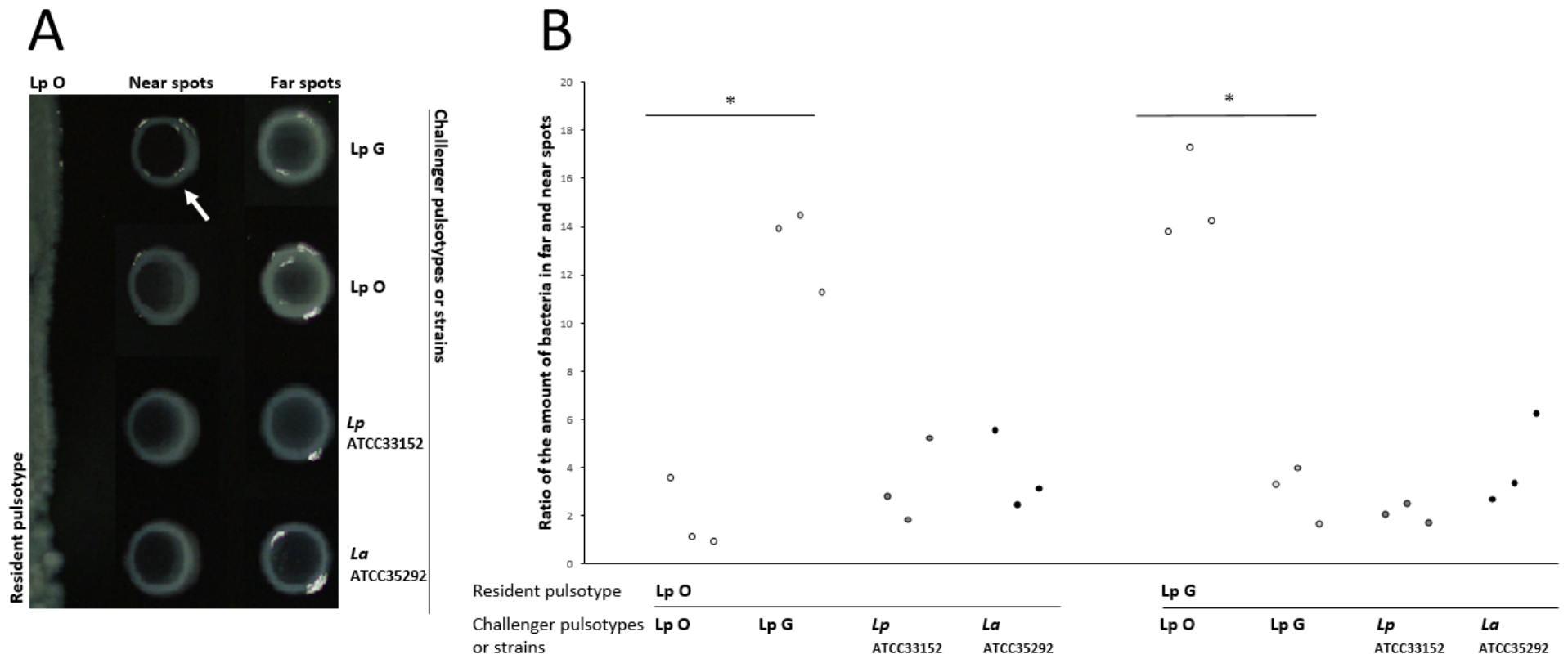


Figure 4

