



HAL
open science

Validation of quantitative real-time polymerase chain reaction for detection of Legionella pneumophila in hospital water networks

M. Sauget, M. Richard, S. Chassagne, D. Hocquet, X. Bertrand, A. Jeanvoine

► To cite this version:

M. Sauget, M. Richard, S. Chassagne, D. Hocquet, X. Bertrand, et al.. Validation of quantitative real-time polymerase chain reaction for detection of Legionella pneumophila in hospital water networks. *Journal of Hospital Infection*, 2023, 138, pp.74-80. <10.1016/j.jhin.2023.06.010>. <hal-04157696>

HAL Id: hal-04157696

<https://hal.science/hal-04157696v1>

Submitted on 9 Jul 2025

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC 4.0 - Attribution - Non-commercial use - International License

Validation of quantitative real-time PCR for detection of *Legionella pneumophila* in hospital water networks

Marlène Sauget^{a,c*}, Marion Richard^{a,c}, Sophie Chassagne^a, Didier Hocquet^{a,b,c}, Xavier Bertrand^{a,b}, Audrey Jeanvoine^a

^a Hygiène Hospitalière, Centre Hospitalier Universitaire de Besançon, Besançon, France

^b Chrono-environnement, Université de Franche-Comté, CNRS, Besançon, France

^c Centre de Ressources Biologiques, Centre Hospitalier Universitaire de Besançon, Besançon, France

* Corresponding author : Marlene Sauget

Hygiène Hospitalière, Centre Hospitalier Universitaire, 3 boulevard Fleming, Besançon, Cedex 25030, France.

Email: msauget@chu-besancon.fr

Tel: + 33 3 70 63 21 35 Fax: + 33 3 70 63 21 27

Running title: qPCR detection of *L. pneumophila*

Word Count: 2573

Keywords: *Legionella pneumophila*; Hospital; Hot water network; Sanitary monitoring; qPCR action thresholds; rapid screening

1 **ABSTRACT**

2 **Background.** Rapid monitoring of *Legionella pneumophila* (Lp) is essential to reduce the risk
3 of Legionnaires' disease in healthcare facilities. However, culture results take at least eight
4 days, delaying the implementation of corrective measures. Here, we assessed the performance
5 of a qPCR method and determined qPCR action thresholds for the detection of Lp in hospital
6 hot water networks (HWNs).

7 **Methods.** Hot water samples (n=459) were collected from a hospital HWNs. Lp were
8 quantified using iQ-Check[®] Quanti real-time PCR Quantification kits (Bio-Rad) and the
9 results were compared with those of culture. qPCR thresholds corresponding to the culture
10 action thresholds of 10 and 1,000 CFU/L were determined on a training dataset and validated
11 on an independent dataset.

12 **Results.** Lp concentrations measured by culture and qPCR were correlated for both the
13 training dataset (Spearman's correlation coefficient $\rho = 0.687$, p-value < 0.0001) and the
14 validation dataset ($\rho = 0.661$, p-value < 0.0001). Lp qPCR positivity thresholds corresponding
15 to culture action thresholds of 10 CFU/L was 91 genome units (GU) per liter (sensitivity,
16 86.4%; negative predictive value – NPV, 93.3%) and that corresponding to culture action
17 thresholds of 1,000 CFU/L was 1,048 GU/L (sensitivity, 100%; NPV, 100%).

18 **Conclusion.** Detection of Lp by qPCR could be implemented with confidence in hospitals as
19 a complement to culture in the monitoring strategy to speed up the implementation of
20 corrective measures.

21 1. INTRODUCTION

22 Legionnaires' disease (LD) is acquired by inhalation of aerosols of water contaminated with
23 *Legionellae*. More than 90% of cases are related to the species *Legionella pneumophila* [1-4].
24 The incidence of LD has increased at a similar rate in France, Europe and the United States
25 since the early 2000s [3, 5-8]. In 2021, the incidence in France and in Europe was 3.0 and 2.4
26 cases per 100,000 inhabitants, respectively [9]. Two major sources of contamination are the
27 hot water networks (HWNs), especially through showers, and cooling towers [1]. Since
28 *Legionellae* often contaminate HWNs in healthcare facilities, monitoring of these pathogens
29 is essential to reduce the risk of patient contamination [10, 11].

30 In France, the sanitary monitoring of the HWNs is regulated by legislation that defines the
31 frequencies, culture action thresholds (CAT) and methods for the detection of *Legionellae*
32 [12]. The conventional culture method (the results of which are expressed as number of
33 colony forming units per liter, CFU/L) is required by law and should be carried out according
34 to the Standard [13]. In Europe, the culture method is also the reference for detection and
35 quantification of *Legionellae* in environmental samples [11, 14]. However, culture results take
36 at least 8 days, delaying the implementation of corrective measures. Therefore, other methods
37 based on molecular detection, such as quantitative real-time polymerase chain reaction
38 (qPCR), can provide faster results [11, 15, 16]. Hence, the standard NF T90-471 defines the
39 use and validation modalities of the qPCR method (the results of which are expressed as
40 number of genome units per liter, GU/L) for the detection and quantification of *Legionellae*
41 [17].

42 Depending on the immune status of the hospitalized patients, legislation sets CAT for
43 hospitals at 10 CFU *L. pneumophila*/L (CAT₁₀) or 1,000 CFU *L. pneumophila*/L (CAT₁₀₀₀)
44 [12]. However, the poor correlation between culture and qPCR results has hindered the
45 definition of action thresholds for qPCR. This is probably because molecular methods detect

46 DNA without distinguishing between live, viable but non-culturable (VBNC), or dead cells.
47 As environmental factors (*i.e.*, chemical disinfection, heat treatment, and starvation) increase
48 the proportion of VBNC and dead cells, qPCR results can be overestimated. Therefore,
49 discrepancies in *Legionellae* concentration between culture and qPCR have been reported in
50 high temperature water [18]. While the culture approach quantifies CFU, qPCR measures GU
51 from dead and live bacteria, providing a different information [15, 18-22].

52 At the University Hospital of Besançon (UHB, France), *Legionellae* persistently contaminates
53 three out of five HWNs that must be regularly monitored to control the risk of nosocomial
54 LD. In order to quickly implement corrective measures for patient protection, we
55 implemented qPCR in the UHB water monitoring strategy in addition to culture. The
56 objectives of this study were (*i*) to verify on site the performance of the standardized qPCR
57 method, (*ii*) to determine appropriate qPCR action thresholds, and (*iii*) to propose the
58 implementation of qPCR as a complement to culture for the detection of *L. pneumophila* in
59 hospital HWNs.

60

61

62 2. MATERIALS AND METHODS

63 **Study setting and water sampling.** The 1,400-bed UHB consists of five buildings,
64 each with its own HWN. We collected one-liter hot water samples in duplicate (one sample
65 for culture and one sample for qPCR analysis) from the five HWNs over a two-year period
66 between January 2020 and December 2021. Sampling points were defined according to the
67 UHB water monitoring strategy. Water was sampled according to the French guidelines (*i.e.*,
68 after removal of faucet accessories and a pre-flush of water until the temperature has
69 stabilized and within 2 to 3 minutes) [23].

70 ***Legionellae* detection and quantification by culture.** We detected and quantified
71 *Legionella* spp. and *L. pneumophila* by according to the French technical guideline [13].
72 Briefly, 200 μ L of water were plated on glycine–vancomycin–polymyxin–cycloheximide
73 (GVPC) agar (Thermofisher Oxoid, Dardilly, France). In addition, 10 and 100 mL of water
74 were filtered through 0.45- μ m membranes (Merck, Darmstadt, Germany), treated with pH 2.0
75 KCl-HCl buffer for five minutes, and then placed on GVPC agar and incubated at $36 \pm 2^\circ\text{C}$
76 for eight to eleven days. Colonies with *Legionellae* characteristics were subcultured on BCYE
77 agar with and without L-cysteine (Thermofisher). Colonies growing only on BCYE agar with
78 L-cysteine were identified as *Legionellae*. Latex agglutination test (Bio-Rad, Marne-la-
79 Coquette, France) was used to identify *L. pneumophila*. Quantification results were expressed
80 in CFU/L according to the French technical guideline [13].

81 ***Legionellae* detection and quantification by qPCR.** DNA was extracted after
82 concentration of the water samples. For this purpose, 1 L of water was filtered through a 0.4-
83 μ m polycarbonate membrane (VWR International, Fontenay sous-Bois, France) according to
84 the recommendations of French Standard [17]. DNA was extracted from the filtration
85 membrane using the AQUADIEN™ extraction kit (Bio-Rad), according to the manufacturer's
86 recommendations. Briefly, the filtration membrane was placed in lysis buffer for 15 min at

87 95°C with stirring for a thermal and a chemical lysis, then DNA was purified on columns and
88 eluted in 100 µL of buffer. We amplified DNA with the iQ-Check® Quanti *Legionella* spp.
89 and *L. pneumophila* real-time PCR Quantification kits (Bio-Rad) using a CFX96™ Touch
90 Deep well Real-Time PCR Detection System (Bio-Rad), according to the manufacturer's
91 recommendations. Results were interpreted using the CFX Manager™ Software - IDE (Bio-
92 Rad) and expressed in GU/L.

93 **Validation of the qPCR kits.** According to the standard recommendations, the
94 validation for qPCR kits consisted of checking and evaluating five points: (i) calibration
95 curve, (ii) detection limit, (iii) quantification limit, (iv) extraction yield, and (v) robustness
96 [17]. To check the calibration curve, five dilution ranges of four concentrations levels were
97 performed under reproducible conditions (*i.e.*, at different days and/or by different operators)
98 using the standard solutions of the qPCR kits. The detection limit was determined in duplicate
99 on ten independent dilutions at 5 GU per assay (as recommended by the Standard). The
100 accuracy of the quantification of limit was evaluated at the lowest concentration of the
101 dilution range. To determine the extraction yield, we compared *L. pneumophila* quantification
102 after direct lysis of a *L. pneumophila* suspension at 10⁹ GU/ml in triplicate with that obtained
103 after filtration and extraction of two suspensions at 10³ and 10⁵ GU/L in quadruplicate.
104 Robustness was assessed by testing with water from a *Legionellae*-free HWN.

105 **Statistical analysis.** The correlation between culture and qPCR results was evaluated
106 by calculating the Spearman coefficient correlation (ρ) using MedCalc software (version
107 20.106, Ostend, Belgium). To determine qPCR action thresholds according to the CAT₁₀ and
108 CAT₁₀₀₀ defined by French legislation, receiver operating characteristic (ROC) curves were
109 calculated (MedCalc v 20.106) by using *Legionellae* quantification on a training data set
110 consisting of water samples collected between January and December 2020 [12]. The validity
111 of the qPCR thresholds was verified using an independent validation data set consisting of

112 water samples collected between January and December 2021. A p -value < 0.05 was
113 considered significant. Sensitivity, specificity, positive and negative predictive values of the
114 qPCR method were calculated from the validation data set using the Wilson score method
115 with OpenEpi 3.03a software (www.openepi.com).

116

117

118 **3. RESULTS**

119 **qPCR validation and comparison with culture.** We validate here a qPCR method to
120 detect and quantify *Legionellae* in HWNs. All checkpoints for the method validation
121 complied with the recommendations of the standard NF T90-471 and define a limit of
122 detection of 80 GU/L and a limit of quantification of 608 GU/L (Supplemental Table 1) [17].
123 A total of 459 water samples were collected in duplicate and analyzed by both culture and
124 qPCR between January 2020 and December 2021. Compared to culture, which requires at
125 least 8 days to obtain results, qPCR provides results 8 hours after sampling. *Legionella* spp.
126 concentrations ranged from <10 to 460,000 CFU/L by culture and from < 80 to 6,190,000
127 GU/L by qPCR with a median concentration of < 10 CFU/L and < 80 GU/L for culture and
128 qPCR, respectively. *L. pneumophila* concentrations ranged from <10 to 460,000 CFU/L by
129 culture and from < 80 to 90,700 GU/L by qPCR, with a median concentration of <10 CFU/L
130 and 85.2 GU/L for culture and qPCR, respectively.

131 Table 1 details the correlation between culture and qPCR results. *L. pneumophila*
132 concentrations measured by the two methods are correlated for both the training set (2020, ρ
133 = 0.687, $p < 0.0001$) and the validation set (2021, $\rho = 0.661$, $p < 0.0001$). These correlations
134 were similar between the two time periods ($p = 0.6293$). Results of the two methods were
135 correlated in Building 1 and Building 2 ($\rho = 0.734$ and 0.501 , $p < 0.0001$, Table 1). However,
136 this correlation differed between these two buildings (difference = 0.233, $p = 0.0002$) (Table
137 1), presumably because their HWNs consisted of a semi-instantaneous production system,
138 either looped for Building 1 and non-looped for Building 2. A looped hot water network
139 prevents water stagnation, unlike a non-looped hot water network where the risk of dead legs
140 is more significant. As a result, the median T_{\max} within the study period in the HWN of
141 Building 1 varied from 51.0°C to 53.6°C and that of Building 2 varied from 58.8°C to 59.2°C.
142 In UHB, for the three temperature ranges tested, results of the two methods were correlated

143 but the ρ values decreased from 0.871 to 0.352 when the temperature increased from $< 50^{\circ}\text{C}$
144 to $> 60^{\circ}\text{C}$ ($p \leq 0.0004$; Table1), in line with previous results [15]. Moreover, the correlations
145 differed between the three temperature ranges (differences between 0.200 and 0.519, $p \leq$
146 0.0003) (Table 1). For *Legionella* spp. the correlation between the two methods was to $<$
147 0.256 ($p < 0.0387$).

148 **Validation of qPCR thresholds for *L. pneumophila* CAT₁₀ and CAT₁₀₀₀.** In France,
149 CAT are regulated and determined only for *L. pneumophila* according to the LD risk for the
150 patients, with CAT₁₀₀₀ being the most used threshold. For high-risk immunocompromised
151 patients, *L. pneumophila* concentration should be below the limit of detection of culture
152 method (*i.e.*, < 10 CFU/L) [12]. Because the risk of LD due to non-*pneumophila* *Legionellae*
153 is low and as the monitoring of *L. pneumophila* contamination is the key measure for
154 controlling LD risk in healthcare facilities, establishing a qPCR threshold for *Legionella* spp.
155 was less useful [11, 12]. Threshold values of qPCR corresponding to CAT₁₀ and CAT₁₀₀₀
156 were first calculated from the ROC analysis (Supplemental Fig. 1) using a training dataset
157 consisting of 299 water samples and then validated using a validation dataset of 160 water
158 samples collected only from buildings which HWN was contaminated with *L. pneumophila*.
159 As qPCR is a convenient test for *L. pneumophila* screening in water, we prioritized the
160 sensitivity and the negative predictive values (NPV) in the calculation of the threshold values.
161 Table 2 details the performance of the qPCR positivity threshold for CAT₁₀ and CAT₁₀₀₀.

162 For CAT₁₀, a calculated qPCR threshold value of 91 GU/L had a sensitivity value of
163 92.9% (95% CI, 85.3-96.7) on the training dataset (Table 2). On the validation dataset, this
164 threshold of 91 GU/L had a sensitivity of 86.4%, an NPV of 93.3%, and a negative likelihood
165 ratio of 0.20 (Table 2). For CAT₁₀₀₀, a calculated qPCR threshold value of 1,048 GU/L had a
166 sensitivity to 93.8% (95% CI, 79.9-98.3) with the training dataset (Table 2). This performance

167 was confirmed with the validation dataset, where sensitivity and negative predictive value
168 (NPV) reached 100% and the negative likelihood ratio was zero (Table 2).

169 **4. DISCUSSION**

170 Our data confirmed the difficulty of setting a conversion factor and comparing qPCR
171 and culture results [24, 25]. To date, the comparison of qPCR and culture results is still under
172 debate with conversion factors ranging from 4 to 28 [19, 20, 22, 26]. Such a discrepancy
173 occurs because qPCR detects all *Legionellae* cells (including VBNC and dead cells) in
174 contrast to culture. Therefore, culture underestimates *Legionellae* concentrations, which are
175 overestimated by qPCR. Solutions have been proposed to limit false positives in qPCR:
176 inhibitors of DNA amplification from dead bacterial cells, detection of a precursor 16S rRNA
177 as an indicator for viability, or pre-step of amoebic co-culture to assess VBNC [26-29].
178 However, these methods provide results difficult to interpret as (i) they do not fully remove
179 DNA from dead cells and (ii) do not solve the problem of the VBNC proportion variability
180 [15, 18, 19, 22, 26, 30-32]. Moreover, the weaker correlation between culture and qPCR for
181 *Legionella* spp. could be explained by the difficulty of culturing some non-*pneumophila*
182 *Legionellae* (Table 1) [27].

183 A meta-analysis of the performance of *L. pneumophila* detection by qPCR including
184 16 studies reported a sensitivity of 94% and a specificity of 72%, with an AUC of 0.79 [33].
185 Our optimal qPCR performance for *L. pneumophila* was similar at CAT₁₀ and CAT₁₀₀₀. The
186 qPCR thresholds defined here are of the same order as those defined for culture (91 GU/L vs.
187 10 CFU/L and 1,048 GU/L vs. 1,000 CFU/L). However, we confirm previous studies showing
188 that qPCR detects more positive samples than culture [19-21, 24, 34-36].

189 We propose the implementation of qPCR as a complement to culture for the detection
190 of *L. pneumophila* in water, with threshold values depending on the previous contamination
191 status in hospital HWNs (Fig. 1). In buildings with uncontaminated HWNs, water points are

192 not secured and the rapid implementation of corrective actions is necessary in case of
193 *L. pneumophila* contamination. Therefore, we proposed to use qPCR only for screening with a
194 qPCR positivity threshold of 91 GU/L (Fig. 1). For buildings which HWNs are intermittently
195 and persistently contaminated, we recommend using qPCR considering the contamination
196 results of the previous water monitoring. For sample points that were negative by culture or
197 were contaminated with *L. pneumophila* at a concentration < 1,000 CFU/L at the previous
198 monitoring, we recommend using qPCR and culture in parallel to quickly confirm the
199 CAT₁₀₀₀ by qPCR with a threshold of 1,048 GU/L, and thus quickly take corrective measures
200 without waiting for the culture result. The culture results obtained in a second time, will
201 confirm or not the contamination at that point of use (Fig. 1). We found that qPCR was
202 irrelevant for sample points where previous monitoring showed *L. pneumophila*
203 contamination > 1,000 CFU/L by culture. In this case, corrective measures are already in
204 place and the delay in reporting the results will not affect the actions to be taken as part of the
205 LD risk management strategy (Fig. 1).

206 Our study has some limitations. The qPCR thresholds and performance indicators
207 were defined on the HWNs of a single hospital. The low number of positive samples in the
208 uncontaminated or intermittently contaminated HWNs of the UHB impeded the testing of
209 qPCR thresholds in each HWN. To define standardized qPCR thresholds, inter-site variability
210 assessment is required. We have tested only one of the qPCR kits available, which have
211 different protocols for DNA extraction or PCR preparation. In addition, the reference of the
212 PCR instrument may affect *Legionellae* quantification using the same qPCR technique.
213 Variability may also be due to differences in the PCR tools and *Legionellae* target gene
214 selected [20]. However, even if the interpretation of the numerical results remains
215 controversial, the qPCR for the detection of *Legionellae* is accepted by the Standard and we
216 confirm here its sensitivity and its ability to provide rapid results on a large dataset (n= 459).

217

218 **5. CONCLUSION**

219 We reported here a high sensitivity and NPV of qPCR for detection and quantification
220 of *L. pneumophila*. Thus, qPCR is a useful tool for a rapid screening and identification of
221 potentially contaminated sampling sites. We suggest that qPCR could be implemented in the
222 annual monitoring strategy to speed up the implementation or adaptation of corrective
223 measures in the contaminated sampling points categorized as “at risk”.

224

225

226 **Funding.** This work was also supported by the Commission des Examens de Laboratoire et
227 des Innovations Analytiques (CELIA) of the University Hospital of Besançon (AJ).

228

229 **Author contributions.** All authors contributed to the design or conduct of the study. MR, SC,
230 and AJ developed the study protocol, collected the samples, and performed or supervised the
231 microbiological analyses. MS performed the statistical analyses. MS, DH, XB, AJ analyzed
232 the data. MS, DH, XB, AJ drafted the manuscript and all authors reviewed and contributed to
233 the manuscript. AJ coordinated the project.

234

235 **Declaration of competing interest.** None of the authors have anything to disclose.

236

237 **REFERENCE**

- 238 [1] Campese C, Descours G, Lepoutre A *et al.* Legionnaires' disease in France. *Med Mal*
239 *Infect* 2015; 45: 65-71. doi:10.1016/j.medmal.2015.01.015.
- 240 [2] Miyashita N, Higa F, Aoki Y *et al.* Distribution of *Legionella* species and serogroups
241 in patients with culture-confirmed *Legionella pneumoniae*. *J Infect Chemother* 2020;
242 26: 411-7. doi:10.1016/j.jiac.2019.12.016.
- 243 [3] Barskey A, Lackraj D, Tripathi PS *et al.* Legionnaire's disease surveillance summary
244 report, United States 2016-2017. Division of Bacterial Diseases, National Center for
245 Immunization and Respiratory Diseases, CDC 2020.
- 246 [4] Khodr A, Kay E, Gomez-Valero L *et al.* Molecular epidemiology, phylogeny and
247 evolution of *Legionella*. *Infect Genet Evol* 2016; 43: 108-22.
248 doi:10.1016/j.meegid.2016.04.033.
- 249 [5] Beaute J, Zucs P, de Jong B. Legionnaires disease in Europe, 2009-2010. *Euro surveill*
250 2013; 18: 20417. doi:10.2807/ese.18.10.20417-en.
- 251 [6] Joseph CA. Legionnaires' disease in Europe 2000-2002. *Epidemiol Infect* 2004; 132:
252 417-24. doi:10.1017/s0950268804002018.
- 253 [7] Joseph CA, Ricketts KD. Legionnaires disease in Europe 2007-2008. *Euro surveill*
254 2010; 15: 19493.
- 255 [8] European Centre for Disease Prevention and Control. Legionnaires' disease - Annual
256 epidemiological report for 2020. [https://www.ecdc.europa.eu/en/publications-](https://www.ecdc.europa.eu/en/publications-data/legionnaires-disease-annual-epidemiological-report-2020)
257 [data/legionnaires-disease-annual-epidemiological-report-2020;](https://www.ecdc.europa.eu/en/publications-data/legionnaires-disease-annual-epidemiological-report-2020)
- 258 [9] Santé publique France. Légionellose : bilan des cas notifiés en France en 2021.
259 [https://www.santepubliquefrance.fr/maladies-et-traumatismes/maladies-et-infections-](https://www.santepubliquefrance.fr/maladies-et-traumatismes/maladies-et-infections-respiratoires/legionellose/articles/legionellose-bilan-des-cas-notifies-en-france-en-2021)
260 [respiratoires/legionellose/articles/legionellose-bilan-des-cas-notifies-en-france-en-](https://www.santepubliquefrance.fr/maladies-et-traumatismes/maladies-et-infections-respiratoires/legionellose/articles/legionellose-bilan-des-cas-notifies-en-france-en-2021)
261 2021

- 262 [10] Van Kenhove E, Dinne K, Janssens A, Laverge J. Overview and comparison of
263 *Legionella* regulations worldwide. Am J Infect Control 2019; 47: 968-78.
264 doi:10.1016/j.ajic.2018.10.006.
- 265 [11] Walker JT, McDermott PJ. Confirming the presence of *Legionella pneumophila* in
266 your water system: a review of current *Legionella* testing methods. J AOAC Int 2021;
267 104: 1135-47. doi:10.1093/jaoacint/qsab003.
- 268 [12] Legifrance. Arrêté du 1er Février 2010 relatif à la surveillance des Légionelles dans
269 les installations de production, de stockage et de distribution d'eau chaude sanitaire,
270 Paris, France.
- 271 [13] NF T90-431. Qualité de l'eau — Recherche et dénombrement de *Legionella* spp et de
272 *Legionella pneumophila* — Méthode par ensemencement direct et après concentration
273 par filtration sur membrane ou centrifugation. 2017.
- 274 [14] ISO 11731. Water quality-detection and enumeration of *Legionella*. Geneva,
275 Switzerland. 1998.
- 276 [15] Joly P, Falconnet PA, Andre J *et al.* Quantitative real-time *Legionella* PCR for
277 environmental water samples: data interpretation. Applied and environmental
278 microbiology 2006; 72: 2801-8. doi:10.1128/AEM.72.4.2801-2808.2006.
- 279 [16] Ballard AL, Fry NK, Chan L *et al.* Detection of *Legionella pneumophila* using a real-
280 time PCR hybridization assay. J Clin Microbiol 2000; 38: 4215-8.
281 doi:10.1128/JCM.38.11.4215-4218.2000.
- 282 [17] NF T90-471. Qualité de l'eau — Détection et quantification des *Legionella* et/ou
283 *Legionella pneumophila* par concentration et amplification génique par réaction de
284 polymérisation en chaîne en temps réel. 2015.
- 285 [18] Ditommaso S, Ricciardi E, Giacomuzzi M, Arauco Rivera SR, Ceccarelli A, Zotti
286 CM. Overestimation of the *Legionella* spp. load in environmental samples by

287 quantitative real-time PCR: pretreatment with propidium monoazide as a tool for the
288 assessment of an association between *Legionella* concentration and sanitary risk.
289 Diagnostic microbiology and infectious disease 2014; 80: 260-6.
290 doi:10.1016/j.diagmicrobio.2014.09.010.

291 [19] Collins S, Stevenson D, Walker J, Bennett A. Evaluation of *Legionella* real-time PCR
292 against traditional culture for routine and public health testing of water samples. J
293 Appl Microbiol 2017; 122: 1692-703. doi:10.1111/jam.13461.

294 [20] Yaradou DF, Hallier-Soulier S, Moreau S *et al.* Integrated real-time PCR for detection
295 and monitoring of *Legionella pneumophila* in water systems. Applied and
296 environmental microbiology 2007; 73: 1452-6. doi:10.1128/AEM.02399-06.

297 [21] Collins S, Jorgensen F, Willis C, Walker J. Real-time PCR to supplement gold-
298 standard culture-based detection of *Legionella* in environmental samples. Journal of
299 applied microbiology 2015; 119: 1158-69. doi:10.1111/jam.12911.

300 [22] Ditommaso S, Ricciardi E, Giacomuzzi M, Arauco Rivera SR, Zotti CM. *Legionella*
301 in water samples: how can you interpret the results obtained by quantitative PCR?
302 Molecular and cellular probes 2015; 29: 7-12. doi:10.1016/j.mcp.2014.09.002.

303 [23] FD T90-522. Qualité de l'eau - Guide technique d'échantillonnage pour la recherche
304 de *Legionella* dans les eaux. France. 2006.

305 [24] Hamilton KA, Hamilton MT, Johnson W *et al.* Risk-based critical concentrations of
306 *Legionella pneumophila* for indoor residential water uses. Environ Sci Technol 2019;
307 53: 4528-41. doi:10.1021/acs.est.8b03000.

308 [25] Toplitsch D, Platzer S, Zehner R, Maitz S, Mascher F, Kittinger C. Comparison of
309 updated methods for *Legionella* detection in environmental water samples. Int J
310 Environ Res Public Health 2021; 18. doi:10.3390/ijerph18105436.

- 311 [26] Lee JV, Lai S, Exner M *et al.* An international trial of quantitative PCR for monitoring
312 *Legionella* in artificial water systems. J Appl Microbiol 2011; 110: 1032-44.
313 doi:10.1111/j.1365-2672.2011.04957.x.
- 314 [27] Edagawa A, Kimura A, Miyamoto H. Investigations on contamination of
315 environmental water samples by *Legionella* using real-time quantitative PCR
316 combined with amoebic co-culturing. Biocontrol Sci 2019; 24: 213-20.
317 doi:10.4265/bio.24.213.
- 318 [28] Lizana X, Lopez A, Benito S *et al.* Viability qPCR, a new tool for *Legionella* risk
319 management. Int J Hyg Environ Health 2017; 220: 1318-24.
320 doi:10.1016/j.ijheh.2017.08.007.
- 321 [29] Boss R, Baumgartner A, Kroos S, Blattner M, Fretz R, Moor D. Rapid detection of
322 viable *Legionella pneumophila* in tap water by a qPCR and RT-PCR-based method.
323 Journal of applied microbiology 2018; 125: 1216-25. doi:10.1111/jam.13932.
- 324 [30] Kontchou JA, Nocker A. Optimization of viability qPCR for selective detection of
325 membrane-intact *Legionella pneumophila*. J Microbiol Methods 2019; 156: 68-76.
326 doi:10.1016/j.mimet.2018.12.003.
- 327 [31] Yanez MA, Nocker A, Soria-Soria E, Murtula R, Martinez L, Catalan V.
328 Quantification of viable *Legionella pneumophila* cells using propidium monoazide
329 combined with quantitative PCR. J Microbiol Methods 2011; 85: 124-30.
330 doi:10.1016/j.mimet.2011.02.004.
- 331 [32] Delgado-Viscogliosi P, Solignac L, Delattre JM. Viability PCR, a culture-independent
332 method for rapid and selective quantification of viable *Legionella pneumophila* cells
333 in environmental water samples. Appl Environ Microbiol 2009; 75: 3502-12.
334 doi:10.1128/AEM.02878-08.

- 335 [33] Yin X, Chen YZ, Ye QQ *et al.* Detection performance of PCR for *Legionella*
336 *pneumophila* in environmental samples: a systematic review and meta-analysis.
337 *Annals of clinical microbiology and antimicrobials* 2022; 21: 12. doi:10.1186/s12941-
338 022-00503-9.
- 339 [34] Fisher KE, Wickenberg LP, Leonidas LF *et al.* Next day *Legionella* PCR: a highly
340 reliable negative screen for *Legionella* in the built environment. *J Water Health* 2020;
341 18: 345-57. doi:10.2166/wh.2020.004.
- 342 [35] Kirschner AKT. Determination of viable *Legionellae* in engineered water systems: Do
343 we find what we are looking for? *Water research* 2016; 93: 276-88.
344 doi:10.1016/j.watres.2016.02.016.
- 345 [36] Caicedo C, Rosenwinkel KH, Exner M *et al.* *Legionella* occurrence in municipal and
346 industrial wastewater treatment plants and risks of reclaimed wastewater reuse:
347 Review. *Water Res* 2019; 149: 21-34. doi:10.1016/j.watres.2018.10.080.
- 348
- 349
- 350

351 **Tables and figures captions**

352

353 **Table 1. Correlation between the culture and qPCR method for *Legionellae***
354 **quantification according to the years, the sampling sites, and the temperature range.** All
355 water samples were from the University Hospital of Besançon (UHB, France). The
356 Spearman's correlation coefficient (ρ) was calculated with MedCalc software (v 20.106). A p -
357 value < 0.05 was considered significant. For two of the five buildings, the qPCR and culture
358 results were mostly of 0, making it impossible to calculate ρ .

359

360 **Table 2. Validation of qPCR positivity threshold for the detection of *Legionella***
361 ***pneumophila* according to the culture action threshold.** First, the improved qPCR
362 positivity threshold were defined on a training dataset of samples collected in the hot water
363 network of the University Hospital of Besançon (France) between January 2020 and
364 December 2020. The test was considered as positive if the qPCR result was above the
365 threshold. Sensitivity, specificity, positive likelihood ratio (LR+) and negative likelihood
366 ratio (LR-) were calculated with MedCalc software (version 20.106, Ostend, Belgium).
367 Second, the qPCR thresholds were validated on a dataset of water samples collected only
368 from the buildings whose hot water network was contaminated with *L. pneumophila* between
369 January and December 2021. Sensitivity, specificity, negative predictive values (NPV) and
370 positive predictive values (PPV) of the method were calculated with the Wilson score method
371 using OpenEpi 3.03a software (www.openepi.com).

372

373 **Figure 1. Proposal for the implementation of qPCR (according to standard NF T90-471)**
374 **as a complement to culture (according to standard NF T90-431) for the detection of**

375 ***Legionella pneumophila* in hospital hot water networks (HWN) in the annual sanitary**
376 **control strategy of Legionnaires' disease risks.**

377

378 **Supplemental Table 1. On-site validation of *Legionella* spp. and *Legionella pneumophila***
379 **real-time PCR quantification kit (iQ-Check Quanti, Bio-Rad, Marne-la-Coquette**
380 **France) according to NF T90-471 recommendations.**

381

382 **Supplemental Figure 1. Performance of qPCR method for the detection of *Legionella***
383 ***pneumophila*. qPCR was applied to 299 water samples collected on the hot water network of**
384 **the University Hospital of Besançon (France) between January 2020 and December 2020**
385 **(MedCalc version 20.106, Ostend, Belgium). Receiver operating characteristic (ROC) curve**
386 **were calculated for culture action threshold of 10 CFU/L (A) and 1,000 CFU/L (B).**

387

Table 1. Correlation between the culture and qPCR method for *Legionellae* quantification according to the years, the sampling sites, and the temperature range.

	Study period	Site/range temperature	N	Spearman's correlation coefficient (ρ)	Difference	Significance level (p -value)	Confidence interval 95%	
<i>L. pneumophila</i>	2020-2021	UHB (five buildings)	459	0.676		<0.0001	0.623 - 0.722	
	2020 (training dataset)		299	0.687		<0.0001	0.622 - 0.742	
	2021 (validation dataset)		160	0.661		<0.0001	0.623 - 0.722	
	Comparison of ρ between 2020 and 2021					0.026	0.6293	ND
	2020-2021	Building 1	195	0.734		<0.0001	0.662 - 0.793	
	2020-2021	Building 2	179	0.501		<0.0001	0.382 - 0.603	
	2020-2021	Building 3	12	0.396		0.2028	-0.230 - 0.790	
	Comparison of ρ between Building 1 and Building 2					0.233	0.0002	ND
	Comparison of ρ between Building 1 and Building 3					0.338	0.1285	ND
	Comparison of ρ between Building 2 and Building 3					0.105	0.6999	ND
	2020-2021	< 50°C	128	0.871		<0.0001	0.821 - 0.907	
	2020-2021	50-60°C	235	0.671		<0.0001	0.594 - 0.736	
	2020-2021	> 60°C	96	0.352		0.0004	0.163 - 0.516	
	Comparison of ρ between <50°C and 50-60°C					0.200	<0.0001	ND
Comparison of ρ between 50-60°C and >60°C					0.319	0.0003	ND	
Comparison of ρ between <50°C and > 60°C					0.519	<0.0001	ND	
<i>Legionella</i> spp.	2020-2021		459	0.185		0.0001	0.095 - 0.272	
	2020 (training dataset)		299	0.256		<0.0001	0.147 - 0.359	
	2021 (validation dataset)		160	0.164		0.0387	0.009 - 0.311	
	Comparison of ρ between 2020 and 2021					0.092	0.3292	ND

ND, Non Determined

Table 2. Validation of qPCR positivity threshold for the detection of *Legionella pneumophila* according to the culture action threshold.

Culture action threshold (CFU/L)	qPCR positivity threshold (GU/L)	Dataset (number of samples)	Sensitivity (95% CI)	Specificity (95% CI)	LR+	LR-	PPV (95% CI)	NPV (95% CI)
10	91	Training (299)	92.9% (85.3 – 96.7)	65.6% (59.0 – 71.6)	2.70	0.11	-	-
		Validation (160)	86.4% (73.3 – 93.6)	71.6% (62.8 – 79.0)	3.04	0.20	53.5% (42.0-64.6)	93.3% (86.1-96.9)
1,000	1,048	Training (299)	93.8% (79.9 – 98.3)	78.3% (73.0 – 82.8)	4.32	0.08	-	-
		Validation (160)	100% (67.6 – 100)	83.6% (76.9 – 88.6)	6.08	0	24.2% (12.8 – 41.0)	100% (97.1 – 100)

Figure 1.

