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1     **Experimental human-like model to assess the part of viable *Legionella* reaching**  
2                                   **the thoracic region after nebulization**

3

4     **Short title: Experimental human-like model to assess aerosol deposition**

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17

18     **Keywords:** aerosol deposition, human-like model, viable *Legionella*

19

20

## 21 **Abstract**

22 The incidence of Legionnaires' disease (LD) in European countries and the USA has been  
23 constantly increasing since 1998. Infection of humans occurs through aerosol inhalation. To  
24 bridge the existing gap between the concentration of *Legionella* in a water network and the  
25 deposition of bacteria within the thoracic region (assessment of the number of viable  
26 *Legionella*), we validated a model mimicking realistic exposure through the use of (i) recent  
27 technology for aerosol generation and (ii) a 3D replicate of the human upper respiratory tract.  
28 The model's sensitivity was determined by monitoring the deposition of (i) aerosolized water  
29 and Tc<sup>99m</sup> radio-aerosol as controls, and (ii) bioaerosols generated from both *Escherichia coli* and  
30 *Legionella pneumophila* sg 1 suspensions. The numbers of viable *Legionella* prior to and after  
31 nebulization were provided by culture, flow cytometry and qPCR. This study was designed to  
32 obtain more realistic data on aerosol inhalation (vs. animal experimentation) and deposition at  
33 the thoracic region in the context of LD. Upon nebulization, 40% and 48% of the initial *Legionella*  
34 inoculum was made of cultivable and non-cultivable cells, respectively; 0.7% of both populations  
35 reached the filter holder mimicking the thoracic region in this setup. These results are in  
36 agreement with experimental data based on quantitative microbial risk assessment methods and  
37 bring new methods that may be useful for preventing LD.

38

## 39 **Introduction**

40 *Legionella* (gram-negative bacilli) are ubiquitous in natural and anthropogenic aquatic  
41 ecosystems. They are responsible for severe pneumonia that may be fatal in 30% of cases when  
42 considering nosocomial infections. *L. pneumophila* is, by far, the most frequent species  
43 associated with Legionnaires' disease (LD). The incidence of LD in European countries per million  
44 population increased from 4.3 to 11.5 during 1998–2012 (1,2). A recent publication on drinking  
45 water shows that *Legionella* spp. were responsible for 66% of outbreaks and 26% of illnesses in  
46 the USA between 2011-2012 (3). Surveillance of *Legionella* is based on water sample analysis by  
47 a standardized culture assay (AFNOR T90-431 / ISO 11731) and/or standardized real-time PCR  
48 method (AFNOR T90-471 / ISO 12869). Our previous studies on *Legionella* reservoirs have shown  
49 that whatever the method employed, it is difficult to correlate the concentration of *Legionella*  
50 and the infectious risk probably due to the presence of viable but non-culturable *Legionella*  
51 (VBNC) in water samples, and to a lesser extent, the presence of PCR and culture inhibitors (4–8).  
52 Indeed, at least 14 physiological forms of *L. pneumophila* have been described in the

53 environment (9). Among them, the presence of VBNC forms, such as those deriving from  
54 exponential phase forms (EPFs) and stationary phase forms (SPFs), in water samples contributes  
55 to the number of potentially pathogenic *Legionella* being underestimated. In a previous study,  
56 we have shown that VBNC *Legionella* deriving from these forms were infectious for macrophage-  
57 like cells (7).

58 Moreover, human infection occurs almost exclusively through aerosol inhalation. Aerosols drive  
59 *Legionella* to the lungs. It is well documented that the bacteria multiply within a reprogrammed  
60 *Legionella*-specific vacuole into alveolar macrophages and some other cells of the respiratory  
61 mucosa (10–12). To date, very few studies aimed at evaluating the concentration of *Legionella* in  
62 aerosolized particles have been published. For those available, discrepant results do not favour  
63 their use in the field (13–16). To assess the risk of LD infection, QMRA (Quantitative Microbial  
64 Risk Assessment) methods provide models for aerosol dispersion (17–21). However, these risk  
65 models are based on infectious doses extrapolated to humans from animal inhalation (22–26),  
66 intraperitoneal injection or tracheal instillation studies (27,28).

67 To bridge the existing gap between the concentration of *Legionella* in water distribution systems  
68 and the LD infectious doses for humans, there is a need to propose an original human-like  
69 respiratory model coupled with an experimental setup of *Legionella* aerosol generation. In this  
70 study, we describe a model mimicking realistic conditions of exposure to *Legionella* through the  
71 use of the following:

72 (i) A vibrating-mesh nebulization system, which is a recent technology for aerosol  
73 generation (29,30). Such nebulizers mimic the dispersion of aerosols generated by  
74 various devices (showers, cooling towers) connected to water distribution systems  
75 (see S1 supporting information).

76 (ii) A 3D printed replicate of the human upper respiratory tract (initially optimized from a  
77 plastinated nasal cast) previously developed (30–33) and connected to a filter  
78 mimicking the thoracic region.

79 This study is the first to account for both the physiological status of aerosolized *Legionella* and  
80 the inoculum size that may reach the lungs. Moreover, this article demonstrates the usefulness  
81 of our human-like respiratory model dedicated to *Legionella* aerosol deposition. Finally, new  
82 insights into aerosol dispersion, transport and human exposure to LD are provided.

83

## 84 **Results**

### 85 Development and validation of a respiratory model dedicated to *Legionella* aerosol deposition

86 *Set-up description:*

87 Our study has benefited from the newest technology available on the nebulizer market for  
88 aerosol generation, a vibrating-mesh nebulization (29,30). As shown in Figure 1, the setup was  
89 linked to a pump simulating inspiration and allowing aerosol dispersion from the mesh-nebulizer  
90 between 0.6 and 0.9 mL.min<sup>-1</sup>. The human replicate was connected to the mesh-nebulizer  
91 through an inhalation chamber. The thoracic region (TR) was simulated by a filter holder (FH)  
92 with a polycarbonate membrane. The TR was connected to the human replicate through an  
93 artificial trachea. This anatomically realistic upper airway model maintains the natural curvature  
94 and volume of the upper airway and its original aerodynamic behaviour (31).

95  
96 **Figure 1.** Human-like experimental model inside a glove box. This experimental setup allows the  
97 dispersion and collection of *Legionella* aerosols reaching the filter holder mimicking the thoracic region.  
98 Black arrows indicate the direction of airflow.

99

#### 100 *Safety control*

101 First, the natural heterotrophic flora in the glove box and in the experimental room were  
102 measured. The first nebulization tests were carried out with sterile distilled water (aerosolized  
103 water) and suspensions of *Escherichia coli* strain 039 (*Ec 039*) in order to check the sealing of the  
104 respiratory model and potential contamination of the glove box and the experimental room.

105 After several rounds of optimization, no leaks were visually observed. Ten rounds of nebulization  
106 with sterile distilled water showed that only 0.35 ± 0.1 mL (i.e., 8.8%) of the water was lost.

107 Following the first experiments, only 147 out of 6.2x10<sup>6</sup> CFU (i.e., 0.002%) of the aerosolized *Ec*  
108 *039* were detected in the glove box. This value decreased to 24 out of 9x10<sup>6</sup> CFU (i.e., 0.0003%).

109 *Ec 039* was detected neither in the glove box airlock nor in the experimental room. The sealing  
110 of the experimental setup was also checked through aerosol nebulization of *Lp1 008-GFP*  
111 suspension with only 0.0001% of the initial aerosolized inoculum detected in the glove box.  
112 Therefore, we can assume that water leaks are negligible.

113 The weak presence of endogenous flora in the air of the experimental room and in the glove box  
114 (below the quantification limit of 4 according to NF EN ISO 8199 (34)) indicates that the  
115 manipulations were carried out in good conditions and without interference with the  
116 nebulization experiments (data not shown).

117  
118 *The sensitivity of the model as determined by percentages of aerosol deposition within the*  
119 *experimental setup*

120 We monitored the deposition of aerosolized water and radiolabelled  $Tc^{99m}$  as aerosol controls  
 121 and of bioaerosols (*Ec 039* and *Lp1 008-GFP*). The deposition of aerosols in the filter holder (FH)  
 122 mimicking the thoracic region and of the non-aerosolized suspension in the mesh-nebulizer tank  
 123 were assessed by weighing. The deposition of the aerosol radiolabelled with  $Tc^{99m}$  was visualized  
 124 and quantified by 2D planar scintigraphy (Fig. 2).

125  
 126 **Figure 2.** The sensitivity of the model as determined by 2D planar scintigraphy of  $Tc^{99m}$ . (A) Aerosolization  
 127 in the setup for 5 min. Mesh-nebulizer with inhalation chamber (1), Human replicate (2) and Filter holder  
 128 (3). (B) 2D scintigraphic images recorded over a 2-min period for each element of the setup. Regions of  
 129 interest (ROIs, boxed in red) were delimited on the images with correction of the background using a  
 130 mean of 3 external ROIs (blue squares).

131  
 132 The weight percentage of water, the percentage of radioactivity for  $Tc^{99m}$  and the percentage of  
 133 bacteria aerosolized and reaching the FH (Table 1) were calculated for all nebulization  
 134 experiments (n=40). For each experiment,  $4.0 \pm 0.3$  mL (out of 5 mL in the nebulizer tank) was  
 135 finally aerosolized. The time needed to disperse such a volume was  $5.6 \pm 1.2$  min. Therefore, our  
 136 model simulates total aerosol output between 0.6 and 0.9 mL.min<sup>-1</sup>. As shown in Table 1, no  
 137 significant differences were observed between *Ec 039*, *Lp1 008-GFP* and aerosolized water,  
 138 meaning that the presence of biological particles in the aerosols did not alter their pattern of  
 139 deposition. The percentage reaching the FH, i.e., the thoracic region, varied from 2.4 to 7.4%. By  
 140 contrast, the aerosol labelled radioactively using  $Tc^{99m}$  showed higher deposition in the thoracic  
 141 region (9.4%) compared to the three other weighing methods (water aerosol and bioaerosol  
 142 containing *Ec 039* or *Lp1 008-GFP*). In agreement with our previous studies (29–31), these results  
 143 remained in the same 5-10% range of thoracic deposition. The difference between  $Tc^{99m}$  and the  
 144 other markers is because the radiolabelling method is clearly more accurate and sensitive.

145  
 146 **Table 1. Percentages of water,  $Tc^{99m}$  and aerosolized bacteria reaching the filter holder.**

	Aerosols reaching FH (%)
	Mean $\pm$ SD (Min - Max)
Water (n=10)	5.0 $\pm$ 1.3 (3.5 – 7.4)
<i>E. coli 039</i> (n=10)	5.0 $\pm$ 1.1 (3.4 – 6.9)
<i>Lp1 008</i> (n=20)	4.8 $\pm$ 1.4 (2.4 – 7.2)
$Tc^{99m}$ (n=3)	9.4 $\pm$ 4.0 (5 – 12.8)

147 The percentage reaching the filter holder (FH) was obtained by weighing the polycarbonate  
 148 membrane for Water, *Ec 039* and *Lp1 008-GFP* assays. For Tc<sup>99m</sup> radioactivity quantification, see  
 149 Fig 2. n: number of samples.

150  
 151 Quantification of total (viable and culturable (VC), VBNC and dead cells (DC)) bacteria  
 152 VC bacteria were assessed in the calibrated suspensions (CS) and in the aerosolized fraction  
 153 reaching the FH using colony counts (by culture). The quantification of total bacteria was done  
 154 by qPCR.

155  
 156 **Table 2. Quantification of viable and culturable (VC) cells by culture and total cells by qPCR.**

		CS	FH	% at FH	
<b>Culture</b> Number of VC (CFU) <i>p</i> =0.07	<i>Ec 039</i> (n=10)				
	Mean	9.8 x10 <sup>6</sup>	7.6 x10 <sup>3</sup>	0.14	
	<i>SD</i>	1.7 x10 <sup>7</sup>	8.7 x10 <sup>3</sup>	0.11	
	<i>min</i>	7.3 x10 <sup>5</sup>	6.3 x10 <sup>2</sup>	0.02	
	<i>max</i>	5.7 x10 <sup>7</sup>	2.9 x10 <sup>4</sup>	0.29	
	<i>Lp1 008-GFP</i> (n=20)				
	Mean	2.1 x10 <sup>6</sup>	3.1 x10 <sup>3</sup>	0.23	
	<i>SD</i>	2.4 x10 <sup>6</sup>	3.5 x10 <sup>3</sup>	0.17	
	<i>min</i>	1.6 x10 <sup>4</sup>	6.5 x10 <sup>1</sup>	0.08	
	<i>max</i>	7.3 x10 <sup>6</sup>	1.1 x10 <sup>4</sup>	0.71	
	<b>qPCR</b> Number of bacteria (eq. CFU) <i>p</i> =0.1	<i>Ec 039</i> (n=10)			
		Mean	1.3 x10 <sup>9</sup>	2.4 x10 <sup>6</sup>	1.81
<i>SD</i>		2.3 x10 <sup>9</sup>	3.9 x10 <sup>6</sup>	2.19	
<i>min</i>		3.3 x10 <sup>6</sup>	8.4 x10 <sup>4</sup>	0.01	
<i>max</i>		9.2 x10 <sup>9</sup>	1.4 x10 <sup>7</sup>	6.35	
<i>Lp1 008-GFP</i> (n=20)					
Mean		4.5 x10 <sup>7</sup>	5.9 x10 <sup>4</sup>	0.89	
<i>SD</i>		9.6 x10 <sup>7</sup>	1.8 x10 <sup>5</sup>	1.92	
<i>min</i>		1.7 x10 <sup>4</sup>	8.2 x10 <sup>1</sup>	0.00	
<i>max</i>		5.0 x10 <sup>8</sup>	9.5 x10 <sup>5</sup>	8.88	

157 CS: calibrated suspension (quantification of bacteria in the 5 mL suspension deposited in the  
 158 nebulizer tank). FH: Filter holder (quantification of bacteria reaching FH after nebulization). eq.  
 159 CFU: equivalent CFU. n: number of samples. The percentage of bacteria reaching the FH has  
 160 been calculated for each experiment (10 or 20 experiments for *Ec 039* and *Lp1 008-GFP*  
 161 respectively). The percentages presented in the column “% at FH” (in grey) are the mean, the  
 162 standard deviation (SD), the min and the max of the calculated percentage of bacteria reaching  
 163 FH for each experiment.

164  
 165 As shown in Table 2, the same percentage of VC reaching FH was observed when testing  
 166 suspensions of *Ec 039* (n=10) and *Lp1 008-GFP* (n=20) ( $p=0.07$ ). The quantification of total  
 167 bacteria shows no significant difference between the percentages of total *Ec 039* (1.81%) and  
 168 *Lp1 008-GFP* (0.89%) reaching FH ( $p=0.1$ ).

169  
 170 When using a flow cytometric assay (FCA), the detection limit of which is  $1 \times 10^3$  bacteria/mL, an  
 171 *Lp1 008-GFP* calibrated suspension of  $2 \times 10^6$  CFU/mL was necessary considering that a mean of  
 172 0.89% of total *Legionella* cells can reach the FH (Table 2). Similar flow cytometric profiles (Fig.  
 173 3A) were obtained for all the samples (n=10). A decrease in the percentages of viable cells (VC  
 174 and VBNC) upon nebulization is shown in Table 3.

175 Epifluorescence microscopy was used as a visual control for the FCA results (Fig. 3B).

176  
 177 **Figure 3.** Determination of physiological state of *Legionella* aerosols in calibrated suspension before  
 178 aerosolization. A. Representative analysis by FCA. B. Visual control for FCA by epifluorescence microscopy  
 179 (400x). DC: Dead Cells (red bacteria by microscopy). VBNC: Viable But Not Culturable (double-labelled  
 180 bacteria by microscopy). VC: Viable and Culturable (green bacteria by microscopy).

181  
 182 **Table 3. Effect of nebulization on the physiological forms of *Legionella*.**

FCA (%) ( <i>Lp1 008 -GFP</i> ) (n=10)	VC		VBNC		DC	
	CS	FH	CS	FH	CS	FH
<b>Mean</b>	<b>40.2</b>	<b>33.4</b>	<b>48.2</b>	<b>38.8</b>	<b>11.6</b>	<b>27.8</b>
Standard deviation	3.6	3.6	4.5	3.0	6.9	6.6

183 CS: calibrated suspension. FH: Filter holder (after nebulization). Viable and culturable cells (VC);  
 184 viable but not culturable (VBNC) and dead cells (DC). n = number of samples.

185  
 186 Knowing the mean percentage of each physiological form (by FCA) in the CS and in the FH and  
 187 the total number of cells (eq. CFU by qPCR), we could estimate the number of other forms (Table  
 188 4, deduced from qPCR). In the same way, knowing the number of VC cells (CFU by culture), we  
 189 could estimate the number of other forms (Table 4, deduced from culture). As qPCR is more  
 190 sensitive (as qPCR can detect DNA and DNA fragments of dead cells), a higher percentage of  
 191 *Legionella* reaching the FH (i.e., the thoracic region) is observed but there is no significant  
 192 difference between the two methods ( $p=0.07$ ). Upon nebulization, 40% of the initial inoculum is  
 193 made of cultivable cells (VC) and 48% of viable but not cultivable cells (VBNC). Only a small part  
 194 of both VC and VBNC cells within the initial nebulized inoculum may reach the thoracic region  
 195 mimicked by a filter holder (0.7% of both populations).

196

197 **Table 4. Estimation of the number of physiological forms of *Legionella*.**

<u><i>Lp1 008-GFP</i></u> (n=20)	<u>CS</u>			<u>FH</u>			<u>% at FH</u>	
	<u>FCA (%)</u>	<u>deduced from</u>		<u>FCA (%)</u>	<u>deduced from</u>		<u>deduced from</u>	
		<u>qPCR</u>	<u>Culture</u>		<u>qPCR</u>	<u>Culture</u>	<u>qPCR</u>	<u>Culture</u>
Total cells	<b>100</b>	<b>4.5 x10<sup>7</sup></b>	5.2 x10 <sup>6</sup>	<b>100</b>	<b>5.9 x10<sup>4</sup></b>	9.3 x10 <sup>3</sup>	<b>0.89</b>	0.28
VC	<b>40.2</b>	1.8 x10 <sup>7</sup>	<b>2.1 x10<sup>6</sup></b>	<b>33.4</b>	2.0 x10 <sup>4</sup>	<b>3.1 x10<sup>3</sup></b>	0.74	<b>0.23</b>
VBNC	<b>48.2</b>	2.2 x10 <sup>7</sup>	2.5 x10 <sup>6</sup>	<b>38.8</b>	2.3 x10 <sup>4</sup>	3.6 x10 <sup>3</sup>	0.71	0.23
DC	<b>11.6</b>	5.2 x10 <sup>6</sup>	6.1 x10 <sup>5</sup>	<b>27.8</b>	1.6 x10 <sup>4</sup>	2.6 x10 <sup>3</sup>	2.12	0.68

198 qPCR results are expressed in CFU equivalents (see the Materials and Methods section). Culture  
 199 results are expressed in CFU. FCA results are in percentages. Bold numbers are experimental  
 200 data. Other figures were deduced from these data. CS: calibrated suspensions. FH: Filter holder.  
 201 n: number of samples.

202

203 Extrapolation from French target values

204 The current French target values for *Legionella* control in water networks are alert value: 1x10<sup>3</sup>  
 205 *Legionella*/L and action value: 1x10<sup>5</sup> *Legionella*/L. In this study, our purpose was to evaluate the  
 206 number of *Legionella* that can reach the thoracic region if a concentration of *Legionella* equal to  
 207 the French guideline values was present in the hot water distribution system. Unfortunately,  
 208 considering that we could not aerosolize suspensions with bacterial concentrations lower than  
 209 the detection limit of the FCA method, we extrapolated the numbers of viable *Legionella*  
 210 reaching the FH if theoretical exposures of 1x10<sup>3</sup> or 1x10<sup>5</sup> *Legionella* per litre were considered. In

211 our calculations, to mimic a shower, we considered an exposure of 20 minutes under a  
 212 nebulization rate of 0.6 to 0.9 mL/min (Table 5).

213  
 214 **Table 5. Numbers of viable (VC + VBNC) *Legionella* aerosolized and reaching FH (mimicking**  
 215 **thoracic region) if extrapolated from French target values.**

Theoretical concentration of <i>Legionella</i> in water network (CFU/L)	<b>1.0 x10<sup>3</sup> (a)</b>	<b>1.0 x10<sup>5</sup> (b)</b>
Number of <i>Legionella</i> aerosolized (CS - extrapolated from our model)	26 - 40	2640 - 3960
Number of <i>Legionella</i> reaching FH (extrapolated from our model)	<b>0.2 - 0.3</b>	<b>19 - 29</b>

216 (a) alert value (b) action value (37). Extrapolation was done for an exposure of 20 min at 0.6 to  
 217 0.9 mL/min.

218  
 219 Table 5 shows that a maximum of 0.3 and 29 CFU can reach the FH mimicking the thoracic region  
 220 for the French alert and action values, respectively.

221  
 222 **Discussion**

223 The proliferation of *Legionella* in sanitary hot water systems is controlled through the use of  
 224 heat shock or chlorination treatment. Nevertheless, *Legionella* is currently the aetiologic agent  
 225 most frequently associated with community water system outbreaks (65.6%) in the USA (3). In  
 226 Europe, the number of LD cases is mainly correlated with exposure to cooling towers (58%),  
 227 water systems (26%) and pools (1%) (35).

228 *Legionella* control in water systems is associated with concentration thresholds (Colony Forming  
 229 Units – CFU) that have been empirically defined. In the case of sanitary hot water and for the  
 230 general population, the French regulation has set the alert value for *L. pneumophila* to 1x10<sup>3</sup>  
 231 CFU.L<sup>-1</sup>. For high-risk patients (such as those immunosuppressed), the regulation is not to exceed  
 232 the detection limit of the culture method (36). For cooling towers, the Ministry of Environment  
 233 has defined two target values for *L. pneumophila* (37). The first threshold (1x10<sup>3</sup> CFU.L<sup>-1</sup>) is  
 234 associated with the need to undertake measures to control *Legionella* proliferation (alert value);  
 235 the second (1x10<sup>5</sup> CFU.L<sup>-1</sup>) is correlated with an immediate shutdown of the facility (action  
 236 value).

237 Because infection of humans occurs through aerosol inhalation, in this study, we describe a  
238 model that simulates exposure close to that under realistic conditions. Indeed, our model mimics  
239 (i) the way aerosols are produced by showers or cooling towers (nebulization) and (ii) a human  
240 upper respiratory tract (19,38). Compared to jet nebulizers, vibrating-mesh nebulizers generate  
241 reproducible aerosols of respirable size (see S1 supporting information) and there is a lower  
242 residual volume in the reservoir (39).

243 This study is the first to account for both the physiological status of aerosolized *Legionella* and  
244 the inoculum size that may reach the thoracic region. In a previous study on the characterization  
245 of aerosols containing *Legionella*, we showed that direct nebulization into a low-pressure  
246 cascade impactor (DLPI) had no impact on *Legionella* viability (29). However, in the current  
247 study, because of mechanical constraints, the airborne transport from the nebulizer through the  
248 inhalation chamber, human head replicate and trachea killed a significant portion of the  
249 inoculum (Fig. 3 and Table 3). It is not clear whether this phenomenon is due to the lack of  
250 mucosal layers within the upper respiratory tract replicate. Despite this, our setup is more  
251 realistic than any animal model (40), as we generate *Legionella* aerosols on a human head  
252 replicate. To date, to our knowledge, this model remains the most relevant for such purposes.

253 Extrapolating from our model (Table 5), an exposure of 20 min (with aerosol dispersion between  
254 0.6 and 0.9 mL.min<sup>-1</sup> from the calibrated suspension) with 1x10<sup>5</sup> *Legionella* is likely to carry  
255 approximately 30 viable bacteria to the thoracic region. According to the animal infections  
256 experiments as well as those of Armstrong *et al.* on QMRA (Quantitative Microbial Risk  
257 Assessment) models, 30 bacteria are not sufficient to cause infection to people with a "normal"  
258 immune system. But, according to statistics available, most LD cases are related with  
259 immunosuppressed patients. Therefore, this French action value of 1x10<sup>5</sup> *Legionella*/L  
260 (correlated with an immediate shutdown of the facilities), could be decreased for facilities that  
261 can generate aerosols in contact with immunosuppressed persons.

262 To assess the risk of LD infection, the QMRA method provides models for aerosol dispersion.  
263 Hines *et al.* (22) confirmed that a screening-level exposure assessment approach can yield  
264 applicable data to assess the contribution of water uses to potential *Legionella* exposure.  
265 However, QMRA methods are still hindered by the difficulty of properly assessing both the  
266 emission and exposure factors and the physiological role of the respiratory mucosa. Our model  
267 provides experimental data following nebulization as an exposure factor. However, the current  
268 model does not address deposition at the alveoli, which is the target organ for initiation of  
269 *Legionella* infection. In the same manner, some removal of aerosols is likely to occur between  
270 the upper and lower respiratory pathways. Further experiments using an *ex vivo* human-like

271 model are in progress to investigate the physiological role of the respiratory mucosa in the  
272 *Legionella* infection process. In this model, the filter holder (FH) is replaced by porcine *ex vivo*  
273 lungs.

274 There is still a need to evaluate whether an exposure of 20 minutes during a shower is  
275 comparable (the number of aerosolized bacteria reaching the lungs) to an exposure using our  
276 model of nebulization.

277

## 278 **Materials and Methods**

### 279 Experimental procedure

280 The calibrated suspensions were analysed before and after nebulization in the experimental  
281 model.

282

283 **Experimental procedure.** VC: viable and culturable cells. VBNC: viable but non-culturable cells. DC: dead cells. n:  
284 number of samples.

285

### 286 Bacterial culture, suspensions preparation and characterization

287 An *Escherichia coli* (*Ec 039*) strain and a *Legionella pneumophila* serogroup 1 (*Lp1 008-GFP*) GFP-  
288 modified strain were used (41,42). They were stored at -80°C in Cryobank tubes (Mast  
289 Diagnostic, France). After thawing, *Lp1 008-GFP* and *Ec 039* were plated, respectively, onto BCYE  
290 agar (Buffered Charcoal Yeast Extract, SR0110 C, Oxoid, France) and Luria Broth (LB) agar  
291 supplemented with chloramphenicol (Sigma Aldrich, France) at 8 mg/mL (for GFP plasmid  
292 selection) for 24 h (*Ec 039*) or 72 h (*Lp1 008-GFP*) at 37°C. They were then re-plated onto the  
293 same medium and incubated at 37°C for another 24 h (*Ec 039*) or for 3 days (*Lp1 008-GFP*). These  
294 cultures were then used to achieve a 10-mL calibrated suspension (CS) in sterile normal saline  
295 (0.9% NaCl) water. The final concentrations tested were  $2 \times 10^6$  and  $2 \times 10^5$  CFU/mL for *Legionella*  
296 and  $2 \times 10^6$  CFU/mL for *E. coli*.

297

### 298 Description of the experimental model

299 An original human experimental respiratory model mimicking the upper and lower respiratory  
300 systems was used in this study. The setup was composed of a human resin replicate  
301 corresponding to the entire nasal cavity (nasal fossae, frontal, ethmoid and maxillary sinuses)  
302 without the oral cavity. This replicate was obtained after 3D reconstruction of a human head  
303 after a precise computerized tomography scan (CT-scan) as described in previous studies (31,33).  
304 For this study, the replicate was manufactured in transparent water-resistant and non-porous

305 resin. Endoscopy and qualitative CT-scan observations were performed on the replicate in order  
306 to assess anatomical reproducibility with a human plastinated known model (32). Precise  
307 measurements were compared in critical anatomical regions: ostia and maxillary sinuses.  
308 As shown in Figure 1, the human replicate was linked to a pump (model 420-2902 – 230 VAC, 50  
309 Hz, 0.6 Amp, 1 PH, 0.02 HP, Thermo Scientific, France) simulating inhalation and allowing aerosol  
310 dispersion between 0.6 and 0.9 mL.min<sup>-1</sup> of the calibrated suspensions. To avoid any bacterial  
311 contamination of the pump, an environmental filter (PALL filter BB50TE, PALL Medical, France)  
312 was placed after the thoracic filters. The thoracic region (TR) was simulated by a filter holder  
313 (Filter kit and filter PAD PARI, PulmoMed, France) with a polycarbonate membrane (Membrane  
314 filters Nuclepore™ track etched, Whatman, GE Healthcare, France) that allowed optimal  
315 bacterial recovery (according the standard procedures (AFNOR T90-431 / ISO 11731). Five  
316 millilitres of the calibrated suspension was nebulized in the respiratory model through an  
317 inhalation chamber (ID Tandem). The mesh-nebulizer (eRapid® Nebulizer System by PARI, Cystic  
318 Fibrosis Services, France) used to aerosolize the bacterial suspension is an e-base nebulizer  
319 linked to a small compressor that delivered aerosols with a vibrating membrane technology (see  
320 S1 supporting information). For safety reasons, the model was placed in a glove box (815-PGB  
321 “LA PETITE” GLOVE BOX, Fisher Scientific, France).

322

### 323 Retrieving bacterial aerosols at thoracic region for subsequent analysis

324 To quantify the percentage of bacteria reaching the filter holder (the percentage of bacteria  
325 quantified at the target zone in relation to the inoculum concentration introduced into the  
326 nebulizer reservoir), the polycarbonate membrane (Fig 1) was weighed (1 g = 1 mL of water)  
327 before and after the nebulization. The membrane was placed in 5 mL of BYE (Buffered Yeast  
328 Extract, SR0110 C, Oxoid, France) or LB. After agitation for 30 s the membrane was scraped using  
329 an automatic pipette tip. One millilitre was plated twice on adapted culture medium to count  
330 viable and culturable (VC) bacteria; 1 mL was placed in a 1.5-mL tube for DNA extraction and  
331 qPCR analysis. For *Legionella* only, 1 mL was placed in a flow cytometry tube for flow cytometry  
332 (FCA) and microscopic analyses.

333 The same procedure was applied to the residual volumes of suspension remaining in the full  
334 setup: the volume not nebulized (staying in the nebulizer tank after nebulization) and the  
335 volumes collected from the human replicate, inhalation chamber and artificial trachea.

336

### 337 Validation of model safety and sensitivity

338 After several rounds of experiments to optimize the sealing of the entire model, the lack of leaks  
339 in the experimental setup was checked by applying 10 rounds of nebulization with sterile  
340 distilled water. Leaks were assessed by visual control and weighing by comparing the volume of  
341 water aerosolized from the mesh-nebulizer with the residual volume of water remaining in the  
342 full setup after nebulization. The percentage of water is the weight percentage of the water  
343 reaching the target zone in relation to the volume introduced into the reservoir of the nebulizer.  
344 To evaluate potential contamination of the glove box and the experimental room, a second  
345 round of nebulization (n=10) was carried out with *Ec 039*. During these experiments opened  
346 bacterial agar dishes were distributed inside and outside of the glove box (Fig 2). This procedure  
347 was systematically applied during subsequent nebulization assays.  
348 The dispersion of aerosols in the entire setup was assessed using Technetium 99m ( $Tc^{99m}$ ).  
349 Briefly, 4 mL of the radioactive suspension was aerosolized in the setup for 5 min (Fig 3).  
350 Radioactivity was measured over a 2-min period. Images of the setup were acquired by 2D  
351 planar scintigraphy with a planar gamma camera (resolution 128 × 128) using a single detector  
352 equipped with a low-energy, high-resolution collimator: E-cam camera (Siemens, Germany; 397  
353 mm × 500 mm collimator) (43). A region of interest (ROI) was delimited on the images with  
354 correction of the background using a mean of 3 external ROIs (Fig 3B). All calculations accounted  
355 for the background radiation and physical decay of radioactivity. The results were expressed in  
356 terms of the activity loaded into the nebulizer. These data allowed precise determination of the  
357 amount of radioactivity reaching the filter holder and the different regions of the setup. The  
358 results are expressed as the percentage of  $Tc^{99m}$ , the percentage of the radioactive activity (thus  
359 Becquerel's) reaching the target zone with respect to the radioactivity introduced into the  
360 reservoir of the nebulizer.

361

#### 362 qPCR

363 Quantification was performed using GFP mut2 sequence (44) expression by *Ec 039* and *Lp1 008-*  
364 *GFP*. The forward and reverse primers were, respectively, 5'- AGAGTGCCATGCCCGAAGGT -3' and  
365 5'- AAGGACAGGGCCATCGCCAA -3'. Plasmid DNA was extracted from all samples and a standard  
366 curve was made with the NuCleoSpin Plasmid kit (Macherey-Nagel, France) following the  
367 manufacturer's instructions. qPCR was carried out on an ABI Prism 7500 automate (Applied  
368 Biosystems, France), using the 2X Power SYBR® Green PCR Master Mix (Life Technologies,  
369 France) as follows: initial denaturation for 15 min and a two-step cycle consisting of 15 s  
370 denaturation, 1 min annealing and elongation at 60°C. At the end of each elongation step, the  
371 fluorescence of the incorporated SYBR Green dye was measured. At the end of 45 cycles of

372 amplification, a melting curve program was incorporated to check for any primer dimers or other  
373 non-specific amplification. Each sample was run in duplicate. To express results in CFU  
374 equivalents, a standard curve was done with *Lp1 008-GFP* suspensions ranging from  $2 \times 10^7$  to  
375  $2 \times 10^2$  CFU/mL. The results were analysed using Sequence Detection Software version 1.4 (ABI  
376 7500 System Software, Applied Biosystems, France).

377

#### 378 Flow Cytometric Assay (FCA)

379 As previously described (5–7), FCA profiles of samples were obtained by using a combination of  
380 GFP green fluorescence (viable cells expressing GFP) and propidium iodide (PI) red fluorescence  
381 for cells with damaged membranes. Flow cytometric measurements were performed using a BD  
382 FACSCalibur instrument (Becton Dickinson Biosciences) equipped with an air-cooled argon laser  
383 (488-nm emission; 20 mW). The green fluorescent emission from GFP was collected in the FL1  
384 channel (500 to 560 nm), and the red fluorescence from PI was collected in the FL3 channel (670  
385 nm). A threshold was applied to the FL1 channel to eliminate background signals. Analyses were  
386 performed at a low-flow-rate setting. The results were analysed with Cell Quest Pro software  
387 (Becton Dickinson Biosciences).

388

#### 389 Epifluorescence microscopy

390 FCA samples (20  $\mu$ L) were observed on a Nikon Eclipse Ti-S microscope equipped with a Nikon  
391 Digital Sight camera at a magnification of 400. The acquisition and image processing were done  
392 through Nis-D Element 3.0 software (Nikon).

393

#### 394 Statistics

395 The results are presented as the mean  $\pm$  SD (standard deviation). We determined the statistical  
396 significance of differences between groups using Student's *t*-test or ANOVA analyses. The value  
397 of *p* was considered significant for  $p < 0.05$ . The statistical analysis was performed using StatView  
398 software version 5.0 (SAS Institute Inc., USA).

399

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405

406 **Author Contributions Statement**

407 S.A. and J.P. designed experiments. S.A., J.P., L.L. and N.P. conducted the experiments. S.R. and  
408 F.G. analysed the data and reviewed the manuscript. All authors wrote and validate the  
409 manuscript.

410

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532

### 533 **Figure Captions**

534 **Figure 1.** Human-like experimental model inside a glove box. This experimental setup allows the  
535 dispersion and collection of *Legionella* aerosols reaching the filter holder mimicking the thoracic  
536 region. Black arrows indicate the direction of airflow.

537

538 **Figure 2.** The sensitivity of the as determined by 2D planar scintigraphy of Tc<sup>99m</sup>. (A)  
539 Aerosolization in the setup for 5 min. Mesh-nebulizer with inhalation chamber (1), Human  
540 replicate (2) and Filter holder (3). (B) 2D scintigraphic images recorded over a 2-min period for  
541 each element of the setup. Regions of interest (ROIs, boxed in red) were delimited on the images  
542 with correction of the background using a mean of 3 external ROIs (blue squares).

543

544 **Figure 3.** Determination of physiological state of *Legionella* aerosols in calibrated suspension  
545 before aerosolization. A. Representative analysis by FCA. B. Visual control for FCA by  
546 epifluorescence microscopy (400x). DC: Dead Cells (red bacteria by microscopy). VBNC: Viable  
547 But Not Culturable (double-labelled bacteria by microscopy). VC: Viable and Culturable (green  
548 bacteria by microscopy).

549

550 **Experimental procedure.** VC: viable and culturable cells. VBNC: viable but not culturable cells.  
551 DC: dead cells. n: number of samples.

552

553 **S1 supporting information.** Cumulative distribution of airborne droplets characterized by a low-  
554 pressure impactor (DLPI). *Legionella* nebulization and quantification by qPCR (expressed in % of

555 genetic units reaching each stage of the DLPI, i.e., in % of total airborne *Legionella*). Particles  
556 greater than 10 µm were not plotted.