The transmission mode of Legionella from its source

CURRENT STATUS: POSTED

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DOI:
10.21203/rs.2.15489/v1

SUBJECT AREAS
Applied & Industrial Microbiology General Microbiology

KEYWORDS
Legionella, vapor, aerosol, qPCR, membrane, surface tension
Abstract

Background

Legionella pneumonia has a fatality rate of 28%.

Methods

microscope, fluorescence Quantitative Polymerase Chain Reaction (qPCR), force mathematical analysis.

Results and Conclusion

The transmission mode of Legionella from its source was analyzed by microscope and qPCR. The Legionella removal efficiency by a membrane composed of water molecules was 94.5%, and Legionella had difficulty in penetrating through the surface of the water membrane. A deflection point at the interface between water and air indicated a cluster of Legionella that was bonded to the contact surface by some unknown emplastic media. Force analysis showed that the surface tension of water is 10^6 orders of magnitude larger than the net force from the sum of the buoyancy and the weight of Legionella, and revealed that the surface tension of water is so large that a Legionella bacterium cannot break away from the water surface membrane and escape. The qPCR results showed that no Legionella was found in the air from a Legionella incubator or the Legionella laboratory. The results demonstrate that Legionella cannot be transmitted to people through water vapor or aerosol. The experimental results also indicate that water was able to remove most Legionella bacteria.

Introduction

National Broadcasting Company (NBC) News reported an outbreak of Legionnaires’ disease in New York City in the USA on August 3, 2015, which resulted in seven deaths. Legionella pneumonia has a fatality rate of 28% (Stout et al., 2007). Therefore, it should be a public health priority to perform additional studies and gather a greater amount of data on Legionnaires’ disease (NBCnews, 2015; Jun Li et al., 2009, 2013, 2017, 2018a, 2018b; Shengkun Dong et al., 2017, 2018).

Inhalation of aerosols containing Legionella spp. is presumed to be the primary means of acquiring Legionellosis. Aerosolized water that can cause infection comes from sources such as cooling towers
and evaporative condensers (Osawa et al., 2014), showers (Breimen et al., 1990), ice-making machines (Graman et al., 1997), refrigerated cabinets, whirlpool spas (Coetzee et al., 2012; Campese et al., 2010), hot springs (Kurosawa et al., 2010), fountains (Palmore et al., 2009), and dental equipment (Kadaifciler et al., 2014). The most common source of Legionnaires' disease outbreaks is cooling towers. Legionella becomes airborne and is transmitted via respiratory droplets containing the bacteria. Upon inhalation, the bacteria can infect alveolar macrophages (Leonardo et al., 2012; Sahid et al., 2013). A previous study showed that Legionella pneumophila could spread at least 6 km from its source by air (Nguyen et al., 2006).

Cohesive forces act on water molecules to create surface tension, which holds together a layer of water molecules on top of more loosely connected water molecules so that this layer behaves like an elastic membrane. The objectives of this study were to investigate if Legionella can break out of a surface layer of water molecules that form a membrane, and spread with vapor or aerosol.

**Materials And Methods**

An optical microscope was used (LEICA DM 5000M, Germany), with 3”×1”×1.0 mm microscope slides, and 18×18 mm cover glasses (Fisher Scientific, USA). Legionella pneumophila subsp. (ATCC® 33152TM) was obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA). Legionella was cultured overnight using #1099 Broth (casamino acids-yeast extract (CYE) buffered) at 37 °C for 12 h.

Legionella was confirmed by a polymerase chain reaction (PCR) assay based on the protocol of Van der Zee (Van der Zee et al., 2002). The forward primer (5’-GAAAATAAAGTAAAAGGGGAAGCC–3’) and reverse primer (5’-ATCAATCAGACGACCAGTGTATTC–3’) were designed according to the 16S rRNA gene sequence in order to amplify a 100-bp DNA fragment specific for Legionella species. The fluorescent probe was designed with 5’-fluorescein-CE-phosphoroamidite (FAM) on one end, and the sequence is: 5’FAM-AGGCGTTGTTGTATTGCCAAGTGGTT-BHQ1–3’. The primers were purified with ULTRAPAGE, which consists of a combination of polyacrylamide gel electrophoresis (PAGE) separation and mass spectrometry (MS) analysis. High-performance liquid chromatography (HPLC) was used to purify the fluorescent probe.
The quantitative PCR (qPCR) reaction mixture, 20 µL final volume, contained 10 µL of 2×SuperRealPreMix Plus, 0.6µL of the forward primer, 0.6 µL of the reverse primer, 0.4µL of fluorescent probe, 1µLof DNA template, and 7.4 µL of RNase-free ddH₂O (Tiangen, China). The quantitative PCR equipment used CFD-3220, DNA Engine Opticon, MJ Research Inc., USA. The real-time qPCR protocol is as follows: ① samples were preheated for 15 min at 95 °C, ②samples were heated to denature for 3 seconds at 95 °C, ③samples were cooled to extend for 30 seconds at 60 °C, ④read fluorescence strength, ⑤cycle 40 times from ② to ④. A negative control was also analyzed in each real-time qPCR run. Amplified DNA was detected by agarose gel electrophoresis with Gene Green nucleic acid staining (RT210) (Tiangen, China). The test was performed on all colonies that tested positive with the 2×SuperReal PreMix Plus (probe) (FP206) (Tiangen, China).

The forward and reverse primer, and fluorescent probe were purchased from Sangon Biotech (Shanghai) Co., Ltd. DNA sequencing of the specific PCR DNA fragments was performed by Sangon Biotech (Shanghai) Co., Ltd.

Results

3.1 Legionella observation by microscope at t = 0, 1, 2 min

An optical microscope that automatically captured images at a rate of one image per minute was used to investigate the movement of Legionella. Figure 1(a) shows the first image of Legionella at the initial time t = 0 min, where each dot represents one Legionella bacteria. In the upper left corner, the wavy line represents the interface between water and gas. Atmospheric gas was present on the left side of the interface, and a solution of water and Legionella was on the right side of the interface. At the interface, there was a single row of Legionella at t = 0 min. The water in the observed Legionella solution was continuously vaporizing under the influence of the ambient temperature (22 °C) in the laboratory. In the upper left corner, the wavy line represents the interface between water and gas, and runs from left to right. Figure 1 shows that Legionella was evenly distributed in solution, and after counting under the microscope, it was determined that there were 775 Legionella bacteria in this image. The actual size of this image is 0.394667×0.2960 mm. To investigate the mobility of Legionella, the microscope was focused on the interface line.
Figure 1 (b) was captured at t = 1 min and shows that most of the *Legionella* was absorbed on the right side of the interface line between water and gas; only 19 *Legionella* bacteria remained on the left side of the interface line. Microscopic observation indicated that there were three clusters of *Legionella* on the left side of the interface line, showing that the clusters of *Legionella* bacteria could not have been removed by water. The mechanism that caused these phenomena was still not clear. It was presumed that *Legionella* was absorbed on the surface of incompletely dissolved broth (#1099 broth, CYE) or certain emplastic media. Five clusters of *Legionella* (24 *Legionella* in total) were found to remain on the right side of the interface, as shown in Figure 1 (b). Considering that 19 *Legionella* remained on the left side of the interface, 43 *Legionella* in total were not removed out of 775 *Legionella* in Figure 1 (b). Hence, the *Legionella* removal efficiency was 94.5%, with only clusters of *Legionella* remaining. A single *Legionella* bacterium could be 100% removed by water, showing that the water surface tension is stronger than the movement force of a single *Legionella* bacterium. The circles on the upper left side of Figure 1 (b) came from an unclear microscope lens, and were not *Legionella* because they appeared in every image.

Figure 1 (c) was captured at t = 2 min and shows that the interface moved towards the right side. Comparing Figure 1 (c) to Figure 1 (b), the moving distance of the interface was 0.060639 mm in one minute. Therefore, the moving rate of water \( V_t = 1 \) was 0.060639 mm min\(^{-1}\). Figure 1 (c) also showed that there was an inflection in the interface when it met a cluster of *Legionella*, suggesting that the force of a cluster of *Legionella* was stronger than the water surface tension.

3.2 *Legionella* observation by microscope from t = 12 to 23 min

The lens of the microscope was maintained at the same position to perform observations from t = 12 to 23 min. Images were automatically captured every minute. Figure 2 (a) was captured at t = 12 min. Clusters of *Legionella* that were not removed by water were labeled from 1 to 9. Clusters that were labeled with 1, 2, 3, 4, 5, 6, 7, 8, and 9 contained 2, 1, 3, 1, 3, 2, 1, 4, and 1 *Legionella* bacteria bacterium that remained, respectively. Thus, 18 *Legionella* bacteria in total were not removed in Figure 2 (a). There was a minimum of two rows, an average of 3.5 rows, and a maximum of five rows.
of *Legionella* at the interface in Figure 2 (a). The quantity of *Legionella* at the interface at \( t = 12 \text{ min} \) was 3.5 times more than that at \( t = 1 \text{ min} \) because there was less than a row of *Legionella* at the interface at \( t = 1 \text{ min} \).

Figure 2 (b) was captured at \( t = 13 \text{ min} \). Cluster Number 7 of *Legionella* obviously affected the shape of the interface, showing that the force of a cluster of *Legionella* was stronger than the water surface tension. Through calculation, it was determined that the moving rate of water at \( t = 13 \text{ min} \) was 0.044101 mm min\(^{-1}\) \((V_{t=13} = 0.044101 \text{ mm min}^{-1})\), which was less than the moving rate at \( t = 1 \text{ min} \) \((V_{t=1} = 0.060639 \text{ mm min}^{-1})\).

Figure 2 (c) was captured at \( t = 14 \text{ min} \). In Cluster Number 10, two *Legionella* bacteria appeared, as shown in Figure 2 (c), but did not appear in Figure 2 (b). This result showed that the *Legionella* of Cluster Number 10 broke away from the water surface membrane and escaped. The moving rate of water at \( t = 14 \text{ min} \) was 0.038589 mm min\(^{-1}\) through calculation \((V_{t=14} = 0.038589 \text{ mm min}^{-1})\), which was less than the moving rate at \( t = 1 \text{ and } 13 \text{ min} \).

Figure 2 (d) was captured at \( t = 15 \text{ min} \). The moving rate of water at \( t = 15 \text{ min} \) was 0.025726 mm min\(^{-1}\) through calculation \((V_{t=15} = 0.025726 \text{ mm min}^{-1})\), which was less than the moving rate at \( t = 1, 13, \text{ and } 14 \text{ min} \).

Figure 2 (e) was captured at \( t = 18 \text{ min} \). The moving rate of water at \( t = 18 \text{ min} \) was 0.023341 mm min\(^{-1}\) through calculation \((V_{t=18} = 0.023341 \text{ mm min}^{-1})\), which was less than the moving rate at \( t = 1, 13, 14, \text{ and } 15 \text{ min} \). There was a minimum of three rows, an average four rows, and a maximum of five rows of *Legionella* at the interface in Figure 2 (e). The *Legionella* quantity at the interface at \( t = 18 \text{ min} \) was higher than at \( t = 1 \text{ and } t = 12 \text{ min} \), showing that the *Legionella* quantity increased as time increased. Similar to two *Legionella* bacteria that escaped from the interface in Cluster Number 10, two *Legionella* bacteria in Cluster Numbers 11 and 12 also escaped from the interface. Thus, a total of four out of the initial 775 *Legionella* bacteria (0.52%) escaped from the water membrane, suggesting that *Legionella* does not easily break out of a water membrane.
Figure 2 (f) and (g) were captured at \( t = 21 \) and 22 min, respectively. The moving rate of water at \( t = 21 \) min was 0.021219 mm min\(^{-1}\) (\( V_{t=21} = 0.021219 \) mm min\(^{-1}\)), which was less than the moving rate at previous times of \( t = 1, 13, 14, 15, \) and 18 min. There was an average of five rows of *Legionella* bacteria at the interface at \( t = 22 \) min, as shown in Figure 2 (g), suggesting that the quantity of *Legionella* at the interface increased as time increased.

Figure 2 (h) shows the relationship of moving rate and time, suggesting that the moving rate decreased as time increased. This may be caused by increasing quantities of *Legionella* at the interface line, which can prevent water evaporation.

### 3.3 *Legionella* observation by microscope from \( t = 60 \) to 62 min

Figure 3 shows a microscopic view of *Legionella* bacteria from \( t = 60 \) to 62 min. The deflection points in Figure 3 (a) and (b) at the lower right corner show a cluster of *Legionella*. There was a minimum of 5 rows, an average of 9 rows, and a maximum of 13 rows of *Legionella* bacteria at the interface at \( t = 62 \) min, as shown in Figure 3 (c). The quantity of *Legionella* at the interface at \( t = 62 \) min was more than that before 62 min, showing that water can collect *Legionella*. There was very little escape of *Legionella* bacteria from the water membrane even if there was a maximum of 13 rows of *Legionella* bacteria aggregating at the interface line.

These experimental results showed that water could remove most of the *Legionella* bacteria, and very few *Legionella* bacteria could escape from this body of water. Thus, water can be used as a media of collection for *Legionella* and other bacteria, and based on this experiment, washing hands with water can remove most of the bacteria, thereby preventing it from spreading and subsequently maintaining public health.

### 3.4 Observation by microscope of vapor in a water bubble with *Legionella*

A microscope was used to observe vapor in a water bubble containing *Legionella*, as shown in Figure 4. There were some small water blocks, and *Legionella* was found in the large water bubble. Due to the effect of ambient temperature (22 °C), the large water bubble continuously vaporized. The vapor from the interface of the large water bubble membrane was clearly observed through the microscope,
but it is not clear in Figure 4 because vapor has no color and cannot be shown in the image. Figure 4 shows a darker color near the water membrane and a lighter color farther away from the membrane. The color difference was caused by the vapor concentration, which was highest near the membrane and decreased with increasing distance from the membrane.

Almost all of the *Legionella* bacteria remained inside the membrane and did not exit from the water membrane, suggesting that *Legionella* cannot spread through contaminated water in the form of mist, steam, aerosol, or vapor. This result differs from previous studies and is an important finding. Previous reports showed that *Legionella* could spread through aerosol or vapor. A previous study showed that *Legionella* could spread at least 6 km from its source by air (Nguyen et al., 2006).

3.5 qPCR testing

Air samples were taken from the air inside a *Legionella* incubator and the *Legionella* laboratory four times per month in 2 years. The *Legionella* incubator continuously incubated *Legionella* on buffered charcoal yeast extract (BCYE) solid plates and in liquid BCYE medium in test tubes. The caps were removed from the 50-mL test tubes in which *Legionella* was growing in liquid BCYE medium. Seven days elapsed until the caps were put back on the test tubes. The sample points were at the air output of the incubator and window, door, and at the center of the laboratory. A total of 384 samples were obtained and were analyzed using qPCR. No sample was found positive, showing that no *Legionella* could be found in the air of the *Legionella* incubator or *Legionella* laboratory. This result suggests that no *Legionella* escaped out of solution and contaminated the air through any vapor, steam, or aerosol.

Discussion

Our results show that *Legionella* can be removed by water, but cannot escape from water by air, vapor, steam, or aerosol. The possible reasons are analyzed below. Water is a polar molecule and has a bent molecular geometry with two hydrogen atoms on the oxygen vertex. In the following analysis, a water molecule is assumed to have the following characteristics: the H-O-H gas phase bend angle is 104.48°, as shown in Figure 5 (a), and the distance between the O and H is 95.84 pm (Hoy et al., 1979; Campbell et al., 2009). The positive hydrogen ends connect to the negative oxygen end to form a water molecule. The cohesive force among many water molecules leads to surface tension, and
significant energy is needed to break these intermolecular bonds. Surface tension is also defined as the property of a liquid surface that resists an external force. There are no water molecules above the water surface, resulting in a stronger bond between the molecules in the surface than in the internal body of liquid. This surface layer creates a considerable barrier between air and water. 

Water has the greatest surface tension of any other liquid except mercury. Water has a high surface tension of 0.0728 N m⁻¹ at room temperature (20 °C), which is caused by the strong cohesion between water molecules. This phenomenon can be observed when a paper clip is able to float on the surface of water, as shown in Figure 5 (b) (Buzzle, 2015). Water molecules stay close to each other due to the collective action of hydrogen bonds (cohesion) between water molecules (Campbell et al., 2009), and these bonds also affect microorganisms in contact with them, including bacteria.

In biological cells, hydrophilic protein surfaces have a strong attraction to water. To dehydrate hydrophilic surfaces, a great deal of energy is required against the hydration forces that attract moisture to the surface. These forces are very large but rapidly decrease over a nanometer (Chiavazzo et al., 2014). According to a previous study (Chiavazzo et al., 2014), the influence of a water molecule extends to a distance of one nanometer. Figure 5 (c) is drawn based on the radius of a water molecule at 95.84 pm.

The perimeter of a *Legionella* bacterium is 9.42 µm based on the radius of *Legionella* of 1.5 µm. Therefore, there are 7970 water molecules on the sphere of *Legionella* based on a water molecule distance of 1 nm and water molecule radius of 95.84 pm, as shown in Figure 5 (d). For clarity, Figure 5 (d) only shows 96 water molecules (R = 0.075 µm) on the sphere of *Legionella* out of 7970 water molecules. Figure 5 (d) shows that a single water molecule does not possess sufficient force to move *Legionella* away from it because *Legionella* is much larger than a single water molecule, which indicates that *Legionella* cannot spread out of the solution by air, vapor, steam, or aerosol.

The gravity acting on a bacterium is 1.07–1.19×10⁻³ kg m⁻³ (Docin, 2015). Therefore, the gravity acting on *Legionella* is presumed to be the average of that of a bacterium, i.e., 1.13×10⁻³ kg m⁻³. The volume occupied by a *Legionella* bacterium is 14.13×10⁻¹⁸ m³, and the weight is 15.97×10⁻¹⁵ kg
based on the radius of *Legionella* at 1.5μm. The buoyancy acting on a *Legionella* bacterium is 7.98×10⁻¹⁵ kg if half of a *Legionella* bacterium is immersed in water. The surface tension of water at 20 °C is 0.0727 N m⁻¹.

Assuming *Legionella* A is completely immersed in water, and half of *Legionella* B is immersed in water, as shown in Figure 5 (e), according to force analysis, *Legionella* A is affected by the adhesive force $F_{adw}$ from the water molecules, the weight force $F_w$, and the buoyancy $F_b$. The adhesive forces $F_{adw}$ act in all directions around *Legionella* A so that the adhesive force $F_{adw}$ is zero, i.e., $\Sigma F_{adw} = 0$.

The weight force $F_w$ is 15.97×10⁻¹⁵ kg, and the buoyancy $F_b$ is 7.98×10⁻¹⁵ kg. Therefore, the total net force $F_n$ acting on *Legionella* A is 8.08×10⁻¹⁵ kg, and the force direction is downward, meaning that *Legionella* A cannot spread out of the water, as shown in Equation 1:

$$F_n = \Sigma F_{adw} - F_b + F_w = 0 - 7.98 \times 10^{-15} + 15.97 \times 10^{-15} = 8.08 \times 10^{-15} \text{ kg} \tag{1}$$

Where $F_n$ denotes the total net force in kg, $F_{adw}$ denotes the adhesive force from the water molecule in kg, $F_b$ denotes the buoyancy in kg, and $F_w$ denotes the weight force in kg.

Half of *Legionella* B is immersed in the water, and the other half is in the air. The surface tension $F_t$ of water is equal to that of the adhesive force $F_{adw}$ from water molecules minus the adhesive force $F_{adw}$ from air molecules. According to that, the surface tension of water at 20 °C is 0.0727 N m⁻¹, and the force $F_t$ of *Legionella* B from the surface tension of water is 3.42×10⁻⁸ kg. The net force $F_n$ acting on a *Legionella* bacterium is equal to that of force $F_t$ from the surface tension of water minus buoyancy $F_b$ of a *Legionella* bacterium plus weight $F_w$ of a *Legionella* bacterium, as shown in Equation 2 and Figure 5 (e). Equation 2 shows the buoyancy $F_b$, and weight $F_w$ can be ignored because the surface tension $F_t$ of water is 10⁶ orders of magnitude larger than the buoyancy $F_b$ and the weight $F_w$. The net force $F_n$ acting on a *Legionella* bacterium shows that the surface tension $F_t$ of water is so large that a *Legionella* bacterium cannot break away from the surface membrane of water and escape, and *Legionella* B is pulled into the water due to the surface tension $F_t$:
\[ F_n = F_t - F_b + F_w = 3.42 \times 10^{-8} - 3.99 \times 10^{-15} + 15.97 \times 10^{-15} \text{ kg} \] (2)

where \( F_n \) denotes the total net force in kg, \( F_t \) denotes the surface tension of water in kg, \( F_b \) denotes the buoyancy in kg, and \( F_w \) denotes the weight force in kg.

**Conclusions**

*Legionella* bacteria can be easily removed by a membrane composed of water molecules, with a removal efficiency by the water membrane of 94.5%. It is very difficult for *Legionella* bacteria to break out of a water membrane and escape. Water can be used as a collection medium for *Legionella* and other bacteria. Washing hands with water can remove most of the bacteria and prevent it from spreading, thus maintaining public health. *Legionella* cannot be transmitted to people by mist, air, vapor, steam, or aerosol, but through splashing or touching. The real-time qPCR results suggested that no *Legionella* can escape from solution with air, vapor, steam, or aerosol.

**Declarations**

- **Ethical Approval and Consent to participate**
  Not applicable.
  
- **Consent for publication**
  All authors consent for publication.

- **Availability of supporting data**
  Not applicable.

- **Competing interests**
  The manuscript has no any conflict of interest.

- **Funding**
  This project has been supported by the General Research Items of the Natural Science Foundation of Zhejiang Province, China (Grant No. Y5110280); the Key Research Items of Department of Education of Zhejiang Province, China (Grant No. Z201119987); the Natural Science Foundation of China (Grant No. 21876086); and the Primary Research and Development Plan of Jiangsu Province (Grant No. BE2018708).

- **Authors’ contributions**
Authors’ contributions are the same.

- Acknowledgements

The corresponding author would like to thank Professor Thanh Helen Nguyen for help, thank Professor Shaoting Du and Huijun Liu for instrument support, and also thank Denise R. for language editing. This project has been supported by the General Research Items of the Natural Science Foundation of Zhejiang Province, China (Grant No. Y5110280); the Key Research Items of Department of Education of Zhejiang Province, China (Grant No. Z201119987); the Natural Science Foundation of China (Grant No. 21876086); and the Primary Research and Development Plan of Jiangsu Province (Grant No. BE2018708).

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Figures
(a) *Legionella* at \( t=0 \) min

(b) *Legionella* at \( t=1 \) min
Legionella observation by microscope from t=0 to 2 min: (a) Legionella at t=0 min, (b) Legionella at t=1 min, and (c) Legionella at t=2 min.
(a) $t=12\text{ min}$

(b) $t=13\text{ min}$
(g) $t=22\text{ min}$

(h) Relation of moving rate and time

Legionella observation by microscope from $t=12$ to $23\text{ min}$: (a) image of Legionella ($t=12\text{ min}$), (b) $t=13\text{ min}$, (c) $t=14\text{ min}$, (d) $t=15\text{ min}$, (e) $t=18\text{ min}$, (f) $t=21\text{ min}$, (g) $t=22\text{ min}$, and (h) relationship of moving rate and time.
Figure 3

Legionella observation by microscope from $t=60$ to $62$ min: (a) $t=60$ min, (b) $t=61$ min, and (c) $t=62$ min.
Figure 4

Microscopic observation shows Legionella and steam from contaminated water in a water bubble.
Surface tension of water, (a) Water molecule radius and model (Nguyen et al., 2006; Hoy et al., 1979), (b) a paper clip is able to float on the surface of water (Campbell et al., 2009), (c) molecules at the surface form stronger bonds, (d) Legionella bacteria and water molecule based on the actual scale of Legionella and 783 times the size of a water molecule, and (e)
multiple force analysis of Legionella at different positions in water.