Evaluation of timing of re-appearance of VBNC *Legionella* for risk assessment in hospital water distribution systems

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Abstract

Background. *In this study we estimated the presence of Legionella species, viable but non-culturable (VBNC), in hospital water networks. We also evaluated the time and load of Legionella appearance in samples found negative using the standard culture method.*

Methods. *A total of 42 samples was obtained from the tap water of five hospital buildings. The samples were tested for Legionella by the standard culture method and were monitored for up to 12 months for the appearance of VBNC Legionella.*

Results. *All the 42 samples were negative at the time of collection. Seven of the 42 samples (17.0%) became positive for Legionella at different times of monitoring. The time to the appearance of VBNC Legionella was extremely variable, from 15 days to 9 months from sampling. The most frequent Legionella species observed were Legionella spp and L. anisa and only in one sample L. pneumophila srg.1.*

Conclusion. *Our study confirms the presence of VBNC Legionella in samples resulting negative using the standard culture method and highlights the different time to its appearance that can occur several months after sampling. The results are important for risk assessment and risk management of engineered water systems.*

Introduction

Legionella infection is a major public health concern, particularly in healthcare facilities, because the outcome of the disease is conditioned by the patient's susceptibility (1). In Europe 5,851 cases of Legionella Disease (LD) were reported in 2013, with a case-fatality rate (CFR) of 10%. However, the number of fatalities was more than two times higher (57% of the total) among healthcare associated cases than among community acquired cases (25%) (2). In Italy, during the same year, 1,347 cases

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of LD were reported, 44% of which were healthcare-associated cases and the CFR was 10.4% (3).

Infection is transmitted through inhalation of contaminated water droplets and hospital water networks are often identified as the source of infections, most of which are caused by *Legionella pneumophila* serogroup 1. Water temperatures between 20° to 45° promote the growth of the organism and both hot and cold water networks can be sources of infection. *Legionella* may, however, remain dormant in cold water and multiply when the water temperature reaches a suitable level (4).

Shock hyperchlorination and systemic continuous chlorination are both effective procedures to reduce *L. pneumophila* colonization of water sources in the short-term, but they cannot eradicate it completely (5-7).

The Italian Guidelines for Legionellosis Prevention and Control (8, 9) and the Allegheny County Guidelines (10) emphasize environmental monitoring of *Legionella* species for the prevention of hospital acquired *Legionella* pneumonia, even when there are not recorded cases of disease.

The official method for environmental surveillance of *Legionella* spp is the standard culture technique ISO 11731-2:2004 (11) also reported by the *new* Italian Guidelines for Legionellosis Prevention and Control (12). However, the detection of *Legionella* spp by the standard culture method has some limitations. *Legionella* spp has a slow growth rate that can be inhibited by the presence of other microorganisms like *Pseudomonas aeruginosa* (13) and a viable but non culturable (VBNC) form of *Legionella* might be present (14). VBNC is a physiological state in which bacteria are unable to grow on standard culture media, while continuing to retain certain features of the viable cells such as cellular integrity, metabolic activity and virulence (15). Some Authors think that the VBNC state is a part of the normal

life cycle used as a survival strategy of most bacteria that do not form spores in response to environmental stress, such as extreme temperatures, chlorine oxidative stress, and nutrient starvation (16, 17). Heat treatment may abolish culturability without affecting bacterial integrity (18). Chlorine treatment is the main reason because *L. pneumophila* looses culturability (19-22). A concentration of 2 mg/L for one hour can initiate a stress response in *L. pneumophila* and this induces a cellular protective process with the expression of antioxidant proteins, stress proteins and transcriptional regulators, while the expression of major virulence genes is repressed (18). Chlorine does not cause any damage to the membrane integrity and to the nucleic acid structure, suggesting that these cells preserve their viability, and immediately, after disinfection, *Legionella* has been detected in a VBNC state with no culture method (22). All these stressors can make the cells more sensitive, and the atmospheric oxygen during the plating procedure can increase cellular damage inducing loss of culturability (15).

This aspect has an important implication for Public Health, because these cells are able to resuscitate, multiply and preserve virulence characteristics and could cause sporadic infections and even epidemics (17, 23).

Since the recovery of VBNC implies a long cultivation time of environmental samples, this is never carried out in routine surveillance programs, and there is little knowledge on the extent of the phenomenon in hospital water networks. The aim of this study is to estimate the latent period of *Legionella* species in VBNC state by the culture method in order to evaluate whether the resuscitated cells are able to reach loads that represent a real risk for human health. In this study we have also evaluated the *Legionella* appearance time in samples resulting "negative" to the standard culture method at the time of sampling.

Methods

Water samples were collected from hot and cold water networks from selected buildings in one of the oldest and largest Italian teaching hospitals, which consists of 54 different buildings. After two cases of hospital legionellosis occurred between December 2006 and January 2007 (24), in order to prevent and control the presence of *Legionella* in the hospital water system, a special program was implemented and shock hyperchlorination (sodium hypochlorite 20-50 mg/L of free chlorine at distal points for 1-2 h) and continuous chlorination (0.5-1.0 mg/L) were carried out, until today, in 38 buildings found to contain *Legionella* contamination.

In this context, we collected a total of 42 samples from cold and hot water in five days from five buildings named A-B-C-D-E. Buildings were chosen on the basis of high-risk wards in accordance with the National Guidelines for the prevention of nosocomial legionellosis risk (8). The days of sample collection were chosen according to the hospital surveillance plan (5). Due to logistic constraints, in building B and D the sampling occurred for 4 days only. Additionally, only cold water could be sampled in building B. Two out of the five buildings had no continuous chlorination treatment (buildings A and B) while the other 3 (buildings C-D-E) were provided with continuous chlorination treatment plants because of the presence of high risk patients.

At each sampling location, five litres of water from taps and showers were collected in sterile specimen bottles, previously supplemented with sodium thiosulphate at 0.1 mg/L in deionized water, to neutralize residual free chlorine.

All water samples were analyzed for (a) temperature, using a calibrated electronic thermometer placed in the middle of the water stream, (b) residual free chlorine concentrations and (c) pH, using compact photometer (AL250 Aqualytic, Germany) according to the instructions of the manufacturer.

All samples were transferred to the laboratory at room temperature and processed the same day of collection to determine the occurrence and the concentration of *Legionella* according to ISO method (11). Briefly, 5 L of water were filtered (0.45 μm pore size cellulose esters membrane filter, Millipore, Billerica, MA), the retained material was then suspended in 100 mL of the original sample water by vortexing for 5 min in sterile bottles of 250 mL sealed with screw caps. Two aliquots of 300 μL of samples, after heat treatment (50° for 30 min in a water bath) that reduces contamination by other microorganisms, were cultured on Buffered Charcoal Yeast Extract Agar (BCYE) (Oxoid Ltd., Basingstoke, Hampshire, UK). supplemented with L-cysteine (SR0110, Oxoid) and MWY selective supplement (SR0118, Oxoid). The plates were incubated at 36±1° in a humidified environment at 2.5% CO_2 for 10 days. The reading was performed at intervals of 2-3 days until the tenth day of incubation. Presumptive *Legionella* colonies were subcultured on BCYE agar supplemented with L-cysteine and BCYE L-cysteine-free media to test their inability to grow in absence of this amino acid, and then incubated at 36±1° with 2.5% CO_2. Colonies grown on supplemented BCYE were subsequently identified using an agglutination test (Slidex latex test *Legionella*, bioMérieux SA, France) that distinguishes *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2-15 (polyvalent), and *L. anisa.* For the detection of species of non-*L. pneumophila* Legionellae (polyvalent), we used the *Legionella* species Test Kit (Oxoid). Results were expressed as Colony Forming Units/L (CFU/L).

All samples negative for *Legionella* were kept at room temperature and monitored every 15 days for 12 months with the standard culture method, in order to detect

the appearance of VBNC *Legionella*. One positive control sample of viable *L. pneumophila* srg. 1 was stored at the same conditions and analyzed in the same manner than others samples to verify *Legionella* survival. During the study, our laboratory participated in an external quality control to verify the proficiency of the reference cultural method (25).

Results

The mean values of selected physical-chemical parameters measured the same day of sampling are reported in Table 1.

The concentration of free chlorine was between 0.01 ±0.02 and 0.85 ±0.10 mg/L and pH was between 7.32±0.10 and 7.50±0.10 for the building not in chlorination and for those under chlorine treatment respectively.

All 42 samples were negative for *Legionella* at the time of sampling and were then monitored for up to 12 months. Seven out of the 42 samples (17.0%) became positive for *Legionella* at different times. Those samples were obtained from cold water (from 16.5° ±1.7° to 22.7° ±2.9°) from three out of five buildings (A-B-E; Table 2-1). At the time of resuscitation, *Legionella* concentration was always above

10^2 CFU/L and one sample has reached a critical load of 10^4 CFU/L nine months after sampling. The most frequent *Legionella* species resuscitated cells were *Legionella* spp and *L. anisa* and only in one sample *L. pneumophila* srg.1 (Table 2).

The time resuscitation of *Legionella* VBNC was extremely variable, from 15 days to 9 months after sampling (Table 2).

L. pneumophila srg.1 was detected after 15 days from the sampling time in the building E at the concentration of 1,500 CFU/L; *L. anisa* was detected after three and four months at concentrations of 166 CFU/L and 100 CFU/L respectively (building A) and after four months, at the concentration of 5,300 CFU/L, in the building E. *Legionella* spp was detected after six months at the concentration of 1,764 CFU/L in the building A, and after nine months, at the concentrations of 10,789 CFU/L and 832 CFU/L, in the two samples of the building B (Table 2).

In the positive controls, *L. pneumophila* srg. 1 was detected at all times showing an increasing trend of load (Table 2).

Two water samples, respectively from buildings C and B, were positive for *Pseudomonas aeruginosa* with a high load for all the time of the observational study, resulting negative for *Legionella* recovery (Table 2).

Table 1- Physical and chemical characteristics of water on the day of sampling.

Building	Chlorine treatment	Water system	Samples No	Water (°C) Mean (SD)	Free Chlorine (mg/L) Mean (SD)	pH Mean (SD)
A	No	Cold	5	22.7 (2.9)	0.09 (0.08)	7.41 (0.09)
A	No	Hot	5	60.6 (12.1)	0.01 (0.02)	-
B	No	Cold	4	19.5 (3.7)	0.15 (0.06)	7.39 (0.04)
C	Yes	Cold	5	15.4 (0.5)	0.45 (0.21)	7.32 (0.10)
C	Yes	Hot	5	51.4 (3.0)	0.85 (0.10)	-
D	Yes	Cold	4	14.8 (0.5)	0.57 (0.05)	7.50 (0.10)
D	Yes	Hot	4	48.7 (1.9)	0.45 (0.20)	-
E	Yes	Cold	5	16.5 (1.7)	0.72 (0.18)	7.43 (0.11)
E	Yes	Hot	5	42.2 (1.9)	0.37 (0.22)	-

Table 2 - Frequency and time of resuscitation of VBNC *Legionella* species in water samples of the hospital water network. All samples were negative to *Legionella* at the first time of sampling.

Building/ Samples	Chlorine treatment	Water system	Time of resuscitation (month)				Positive samples
			1-3	4-6	7-9	>9	
Positive* control	Yes	Cold	1[#]	1[§]	1[§]	1[§]	-
A	No	Cold	1[a]	2[b,c]	0	0	**3/5**
A	No	Hot	0	0	0	0	0/5
B	No	Cold	0	0	0	2[d,e]	**2/4**
C	Yes	Cold	0	0	0	0	0/5
C	Yes	Hot	0	0	0	0	0/5
D	Yes	Cold	0	0	0	0	0/4
D	Yes	Hot	0	0	0	0	0/4
E	Yes	Cold	1[f]	1[g]	0	0	**2/5**
E	Yes	Hot	0	0	0	0	0/5
Tot. Legionella resuscitation samples			2	3	0	2	**7/42**

Legend: * Viable *L. pneumophila srg.1* detected at the time of sampling; [#]73 CFU/L; [§]>10^{4}CFU/L [a] *L. anisa* (3 month 166 CFU/L); [b] *L. anisa* (4 month 100 CFU/L); [c] *L. spp* (6 month 1764 CFU/L); [d] *L. spp* (9 month 10789 CFU/L); [e] *L. spp* (9 month 832 CFU/L); [f] *L. pneumophila srg.1* (15 days 1500 CFU/L); [g] *L. anisa* (4 month 5300 CFU/L).

Discussion and Conclusions

We report the results of an observational study focusing on the VBNC *Legionella* state in the water system of a large teaching hospital in Italy that uses chlorine strategy to manage and prevent legionellosis risk.

The results of our study show the presence of VBNC *Legionella* in water networks of three out of five buildings monitored that were negative for the detection of *Legionella* at the time of sampling; and ours appears to be the first study of qualitative and quantitative characterization of *Legionella* spp and of the evaluation of timing of reappearance.

In our study *Legionella anisa* and *Legionella* spp were most frequently detected in VBNC state.

L. pneumophila was never detected after one month, while after six months only *Legionella* spp was recovered. In two samples, the time of resuscitation of *Legionella* spp was nine months after the time of sampling. This is higher than the maximum time of eight months reported in the literature (26). Our results also show that low temperature, between 22.7° ±2.9 and 16.5° ±1.7 (Table 1), in the water networks, possibly plays a role in inducing the VBNC state, as we did not obtain any VBNC *Legionella* appearance in hot water.

Some studies have shown that, at 20°, there is a low number of *Legionella* cells, particularly *L. pneumophila* strain, in the biofilms and planktonic phases, probably due to their reduced metabolism at this temperature (27). At 40°, *L. pneumophila* cells are most abundant, at 50° the number of *Legionella* cells is similar to that found at 20° while, at 60° or higher, they completely lose their culturability (27, 28).

Our results are also in agreement with other studies, which show that *Legionella* spp may survive chlorine treatment when in the VBNC state (22, 28).

We have to consider that the storage conditions of the sample, during the monitoring period, were different from the environmental conditions present in the

water network. It is likely that in this last system, the higher availability of nutrients (oxygen, iron, etc.) and biofilm presence could facilitate the re-emergence and re-colonization of *Legionella* in a shorter time, especially in network zones that are not easily reached by the disinfectant. However, our positive control of *L. pneumophila* srg.1 confirmed that also the storage conditions can allow cell proliferation for over twelve months.

With regard to chemical composition, buildings under chlorine treatment showed free chlorine concentrations four times higher than that in the buildings without chlorine treatment (Table 1). Such a presence in the plumbing system may add stress conditions for *Legionella* growth and may influence its detection showing false negatives during hospital environmental surveillance.

It is known that stressful environmental conditions, such as nutrient starvation, saline and oxygen concentration, temperature outside the range of growth, and chlorine treatment can induce *Legionella* to enter into a viable but non-culturable metabolic state (29). Although chlorination and hyperchlorination or other treatments can reduce significantly the level of planktonic *Legionella,* they remain ineffective against sessile communities, and in presence of some species of amoebae like *Acanthamoeba polyphaga* (29, 31).

Our experience shows that the resuscitation of *Legionella* in VBNC state can reach concentrations that are considered a risk for human health (12) and are able to cause sporadic infections or even epidemics.

In any case, VBNC *Legionellae* are probably large portion of the *Legionellae* population in water networks and this form may constitute an unrecognized reservoir for the disease (32).

In general, all pathogens able to be arrested in a VBNC state, are an analytical problem because they can alter the concentrations determined by standard methods and also

by modern molecular techniques (33), but overall represent an unsolved problem in public health and risk assessment.

In fact, *Legionella* risk assessment and related environmental surveillance in hospitals rely on the standard culture techniques, but we have also demonstrated that this method fails when applied to the recovery of VBNC cells or when the *Legionella* concentration in the water sample is lower than the detection limit.

Our experience also highlights the importance of not to neglect *Legionella* in VBNC state when assessing the *Legionella* risk in the nosocomial context, especially if the hospital is equipped with an old water distribution system. This could increase the effectiveness of prevention measures for legionellosis in hospital settings, where the presence of patients with high susceptibility is higher than in the community.

Additionally, it is necessary to examine more carefully the role of some microorganisms, in particular *Pseudomonas aeruginosa,* on *Legionella* viability. There is evidence that signaling molecules (autoinducers, i.e. N-acyl-homoserine lactones) that mediate *Quorum Sensing* in *Pseudomonas aeruginosa,* may modulate gene expression in host cells and may also possess bacteriostatic and formation-suppressing biofilm activity against *Legionella* (34) and their presence in association with biotic and abiotic elements in water networks may predict the success or the failure of *Legionella* detection with standard culture methods (13).

In the light of our results, with the aim to improve risk assessment, it is desirable to develop models to predict the concentrations of the VBNC *Legionella* through the analysis of the characteristic of the water networks (size, age, dead branches, stress factors such as continuous chlorination and other). In order to support legionellosis risk assessment, further evidence is needed to distinguish, in the sample, the proportion of

VBNC *Legionella* state and viable legionella cells when present in concentrations lower than the standard culture method detection limit.

Riassunto

Valutazione del tempo di riattivazione di Legionella VBNC a sostegno dell'analisi del rischio in reti idriche ospedaliere

Introduzione. In questo studio abbiamo valutato la presenza di *Legionella* vitale non coltivabile (VBNC) nella rete idrica di un ospedale. Sono stati valutati anche la concentrazione ed il tempo di rinvenimento di *Legionella* nei campioni risultati inizialmente negativi con il metodo colturale standard.

Metodi. Sono stati collezionati 42 campioni prelevati dalla rete idrica di 5 edifici del nosocomio. I campioni sono stati testati per *Legionella* mediante il metodo colturale standard e monitorati fino a 12 mesi per il rinvenimento di *Legionella* VBNC.

Risultati. Tutti i campioni sono risultati negativi al tempo del campionamento. Sette su 42 campioni (17,0%) sono diventati positivi per *Legionella* in diversi tempi di monitoraggio. Il tempo di rinvenimento di *Legionella* VBNC è risultato estremamente variabile da 15 giorni a 9 mesi rispetto al campionamento. *Legionella* spp e *L. anisa* sono state le specie più frequentemente rinvenute mentre *L. pneumophila* srg.1 è stata rinvenuta solo in un campione.

Conclusioni. Il nostro studio conferma la presenza di *Legionella* VBNC in campioni risultati inizialmente negativi con il metodo colturale e evidenzia la differente tempistica di rinvenimento che può manifestarsi anche dopo diversi mesi dal campionamento. I risultati forniscono un'importante evidenza a supporto della valutazione e gestione del rischio legionellosi correlata a reti idriche artificiali.

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