Evaluation of Legionella pneumophila contamination in Italian hotel water systems by quantitative real-time PCR and culture methods

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Abstract: This study was to define the extent of water contamination by L. pneumophila of Italian hotels using quantitative real-time PCR associated to the conventional culture method. Some hotels (n=19) with different size and age, belonging to an important hotel chain spread on the whole Italian area (Northern, Central, and Southern Italy) were investigated. In each hotel four type of water samples were collected (n=76): hot water samples were taken in 3 different sites of the water distribution system (boiler, bathroom outlet, recycling of hot water) and 1 cold water sample was collected at the inlet of each water distribution system. Water hardness, temperature and residual free chlorine content were also analysed for each sample.
L. pneumophila was detected by culture method in 42% of the hotels (range 2.0x102 - 3.7x104 CFU/L) and in the 21% of these samples the 104 CFU/L value was exceeded. L. pneumophila serogroup 1 was isolated in the 10.5% of the monitored hotels at a level of 102-103 CFU/L. Using

real-time PCR the 74% of the hotels showed a Legionella contamination, with values ranging from 93 to 5.7 x104 GU/L.
The presence of L. pneumophila resulted significantly influenced by water sample temperature (<55-60°C), while no association with water hardness and residual free chlorine content was found. By the comparison of the results obtained with the real-time PCR and the culture method it derives that the molecular method allows to reveal the L. pneumophila presence also in water samples, where the conventional culture method does not show any contamination. Moreover a higher Legionella concentration was observed by quantitative PCR than by conventional culture. Both these findings confirm the high sensitivity of the molecular method and the risk that the conventional method underestimates the legionellae number. In conclusion the characteristics of real-time PCR makes it a promising method as screening followed by the current culture based method in particular when outbreaks of Legionellaire's disease occurs.

Cover Letter

UNIVERSITA' DEGLI STUDI
DEL PIEMONTE ORIENTALE
"Amedeo Avogadro"

DIPARTIMENTO
DI SCIENZE DELL'AMBIENTE E DELLA VITA

Via T. Michel 11
15100 Alessandria

Alessandria, February 18, 2008

I am pleased to submit the manuscript entitled "**EVALUATION OF *Legionella pneumophila* CONTAMINATION IN ITALIAN HOTEL WATER SYSTEMS BY QUANTITATIVE Real-Time PCR AND CULTURE METHOD**".

L. pneumophila is widespread in the aqueous environment and is a common cause of both nosocomial and community-acquired pneumonia. Water systems of large buildings, such as hotels, hospitals and thermal baths are often contaminated by legionellae. After France and Spain, Italy receives the largest number of foreign tourists per year. In 2006 in Italy 145 out of 251 cases of Legionnaire's disease notified to the Istituto Superiore di Sanità were travel associated. Surveying and monitoring of legionellae in the water systems are needed to prevent and control legionellosis. Although *Legionella* spp. are considered ubiquitous, detecting and isolating *Legionella* spp. from environmental water samples can be difficult. This study was to define the extent of water contamination by *L. pneumophila* of some Italian hotels comparing quantitative real-time PCR with the conventional culture method. Water hardness, temperature and residual free chlorine content were also monitored to evaluate the influence of these physico-chemical parameters on *Legionella* presence.

Your sincerely,

Prof. Elisabetta Carraro

Evaluation of *Legionella pneumophila* contamination in Italian hotel water systems by quantitative Real-Time PCR and culture method

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Abstract

This study was to define the extent of water contamination by *L. pneumophila* of Italian hotels using quantitative real-time PCR associated to the conventional culture method. Some hotels (n=19) with different size and age, belonging to an important hotel chain spread on the whole Italian area (Northern, Central, and Southern Italy) were investigated. In each hotel four type of water samples were collected (n=76): hot water samples were taken in 3 different sites of the water distribution system (boiler, bathroom outlet, recycling of hot water) and 1 cold water sample was collected at the inlet of each water distribution system. Water hardness, temperature and residual free chlorine content were also analysed for each sample.

L. pneumophila was detected by culture method in 42% of the hotels (range $2.0x10^2$ - $3.7x10^4$ CFU/L) and in the 21% of these samples the 10^4 CFU/L value was exceeded. *L. pneumophila* serogroup 1 was isolated in the 10.5% of the monitored hotels at a level of 10^2-10^3 CFU/L. Using real-time PCR the 74% of the hotels showed a *Legionella* contamination, with values ranging from 93 to 5.7 $x10^4$ GU/L.

The presence of *L. pneumophila* resulted significantly influenced by water sample temperature (<55-60°C), while no association with water hardness and residual free chlorine content was found.

By the comparison of the results obtained with the real-time PCR and the culture method it derives that the molecular method allows to reveal the *L. pneumophila* presence also in water samples, where the conventional culture method does not show any contamination. Moreover a higher *Legionella* concentration was observed by quantitative PCR than by conventional culture. Both these findings confirm the high sensitivity of the molecular method and the risk that the conventional method underestimates the legionellae number. In conclusion the characteristics of real-time PCR makes it a promising method as screening followed by the current culture based method in particular when outbreaks of Legionellaire's disease occurs.

Keywords: *Legionella*, water, Real-Time PCR, culture method

1. Introduction

Legionellae are facultative intracellular gram-negative bacteria that may cause Legionnaires' disease (legionellosis), Pontiac fever, as well as much more common mild flulike lung infections (Costa et al., 2005). Human infection occurs through inhalation of aerosolized water contaminated with high numbers of *Legionella* bacteria. Although the genus *Legionella* comprises more than 40 species with 64 serogroups, *L. pneumophila* is the most common pathogenic species, accounting for more than 90% of legionellosis cases (Yanez et al., 2005). *Legionella* is ubiquitous in natural and man-made acqueous environments and requires free-living amoebae for its intracellular replication. In appropriate conditions *L. pneumophila* can also survive for long periods as a free organism in low-nutrient environments (Chang et al., 2007). Water systems of large buildings, such as hospitals, thermal baths, hotels, etc., are often contaminated by legionellae (Mouchtouri et al., 2007; Wellingausen et al., 2001). Surveying and monitoring of legionellae in the water systems are needed to prevent and control legionellosis (Delgado-Viscogliosi et al., 2005).

Travel-associated legionella infections are a significant public health problem in Europe. After France and Spain, Italy receives the largest number of foreign tourists per year. In 2006 in Italy 145 out of 251 cases of Legionnaire's disease notified to the Istituto Superiore di Sanità were travel associated. A further 106 cases diagnosed in foreign tourists who travelled to Italy were notified to the Institute by EWGLINET (The European Working Group for *Legionella* infections). A total of 45 clusters were identified, mainly involving hotels and residences located in different Italian regions (Rota et al., 2008).

Although *Legionella* spp. are considered ubiquitous, detecting and isolating *Legionella* spp. from environmental water samples can be difficult (Palmer et al., 1995). Isolation of legionellae from water samples by culture techniques is the method usually preferred, but it has some limitations (Wellinghausen et al., 2001): the fastidious growth requirements of legionellae need prolonged incubation periods (up to 10 days), and frequently an overgrowth by other present microflora can

occur (Catalan et al., 1997; Garcia et al., 2007; Leoni et al., 2001). Moreover the presence of viable but nonculturable (VBNC) *Legionella* has been pointed out in some investigations and different studies reported that non-culturable *Legionella* cells may also cause illness (Miller, 1993; Steinert et al., 1997). The development of more rapid and sensitive alternative methods for the detection and quantification of *Legionella* cells without cultivation is matter of increasing importance for water monitoring (Delgado-Viscogliosi et al., 2005). Molecular methods, such as quantitative real-time polymerase chain reaction (qPCR), offer an efficient approach to enumerate human pathogens using total DNA isolated from environmental samples (Behets et al., 2007). The main advantages of this technique is the ability to detect *Legionella* contamination at very low level. The fast acquisition of results and the easier handling of large sample amounts are a further useful advantage. Therefore the qPCR constitutes a rapid tool for the detection of the bacterium, the recognition of an outbreak, the risk assessment and prevention of the disease spreading (Fiume et al., 2005). Several qPCR assays targeting *L. pneumophila* have been developed during the last years (Ballard et al., 2000; Behets et al., 2007; Joly et al., 2006; Levi et al., 2003; Wellinghausen et al., 2001). The use of qPCR in the direct enumeration of *L. pneumophila* was evaluated in natural water, hospital water and cooling tower water samples (Ballard et al., 2000; Joly et al., 2006; Levi et al., 2003; Wellinghausen et al., 2001). Moreover some authors reported that the interpretation of qPCR could be influenced by the type of water samples (e.g. cooling tower samples, hot water system samples) (Joly et al., 2006).

This study was to define the extent of water contamination by *L. pneumophila* of some Italian hotels comparing the performance of quantitative real-time PCR with the conventional culture method. Water hardness, temperature and residual free chlorine content were also monitored to evaluate the influence of these physico-chemical parameters on *Legionella* presence.

2 Materials and Methods

2.1. Sample collection

A total of 76 water samples were collected at 19 hotels located in 18 Italian towns (**Fig.1**) between October 2006 and February 2007. The hotels investigated have different size, age and belonged to an important hotel chain spread on the whole Italian area (Northern, Central, and Southern Italy). In each hotel four types of water samples were collected: 1 cold water sample (tap water) was collected at the inlet of each hotel water system and 3 hot water samples were taken in different sites of the water distribution system (boiler, bathroom outlets, hot water recycling). In each sampling point water samples were collected in three sterile glass bottles (1 liter each). In order to neutralize the residual free chlorine sodium thiosulfate was added to sterile bottles for *Legionella* spp. determination, while glass bottles without sodium thiosulfate were used for hardness analysis. Water samples were processed within 24 h from collection.

2.2. Plate culture method

Isolation of *Legionella* from water samples was performed by culture according to International Standard method ISO 11731 (ISO, 1998). One-liter samples of water were concentrated by filtration on 0.22-μm-pore-diameter polycarbonate membrane (Isopore, Millipore). After filtration, bacteria collected on the membranes were resuspended in 10 ml of the original sample water, and 0.1 ml of the suspension was spread on a Petri dish containing BCYE agar supplemented with vancomycin, polymyxin B, cycloheximide, and glycine (GVPC medium) (Oxoid Ltd., Basingstoke, Hampshire, United Kindom). The inoculated plates were then incubated from 7 to 10 days at 37°C ± 1°C. Smooth colonies showing a greyish-white colour were counted as suspected legionellae to be confirmed. Random colonies (10-20) of suspected legionellae were subcultured onto BCYE agar and BCYE agar without L-cysteine. The isolated colonies growing only on BCYE agar were identified by an agglutination test (Legionella latex test; Oxoid). This test allows the identification of *L. pneumophila* serogroup 1 from serogroups 2-14 and *Legionella* spp. The results were expressed as CFU/L and the detection limit of the procedure was 100 CFU/L.

2.3. Real-Time PCR

One liter samples of water were concentrated by filtration on 0.22-µm-pore-diameter polycarbonate membrane (Isopore, Millipore). DNA was extracted with a commercially available kit (Aquadien™ Kit, Biorad) according to the manufacturer's instruction: the membrane was transferred into a tube with lysis buffer and treated at 95°C for 5 min. The samples were leaved at room temperature for 20 min. The supernatant (1ml) was collected and DNA was purified by adsorption on a silica column. Finally, DNA was eluted with 100 µl of elution buffer (supplied in the kit) and stored at -20°C until Real-Time PCR analysis.

Quantitative PCR was performed with an iCycler (Biorad) and a commercially available kit (iQ-Check Quanti *L. pneumophila* Kit, Biorad). Samples were examined in duplicate at two concentrations of the template DNA. For each samples 5µl of non diluted DNA or 1:10 diluted DNA were mixed with 40µl of amplification mixture and 5µl of fluorogenic oligonucleotide molecular beacon (MB) probe labeled with FAM 490. The fluorogenic MB probe from iQ-Check Kit targets the *mip* gene (*macrophage infectivity potentiator*) which is highly specific for *Legionella pneumophila* (Ratcliff at al., 1998). Sterilized water (5µl) was used as PCR negative control. For each batch of samples 5µl of 4 different *Legionella pneumophila* DNA standard (Qs1, Qs2, Qs3 and Qs4 supplied in the kit) corresponding to concentrations between 15 and 3 $x10^4$ genomic unit (GU) were used for DNA quantification. The Kit provides a synthetic DNA as a part of the reaction mixture which works as an internal control, to monitor successful DNA amplification in each reaction tube. This control DNA was amplified with a specific probe at the same time as the *Legionella pneumophila* target DNA sequence and detected by a second fluorophore (Texas red 575). The PCR reaction was run for 50 cycles: denaturation step was at 95°C for 15 s, annealing at 57°C for 30s and extension at 72°C for 30s. An initial 15 min denaturation at 95°C and a final

extension at 72°C were used. Data were collected after each annealing step using an excitation wavelength of 490 nm and emission wavelength of 530 nm as specified by Biorad laboratories. iCycler iQ^{TM} software uses the cycle threshold and the positive control fluorescence value to detect the presence and the quantity of *Legionella pneumophila* DNA. For each sample the iCycler iQ^{TM} indicates the final result expressed as number of genome unit (GU) per liter. The detection limit of the procedure was 80 CFU/L and the quantification limit was 480 GU/L.

2.4. Physical and chemical analyses

Water temperature (thermometer HI 9060, Hanna Instrument) was determined at the time of sample collection. Standard techniques were used to measure water hardness (method 2040; IRSA-CNR, Rome, Italy). The residual free chlorine content (colorimetric method; Aquaquant^{TM}; Merck, Darmstadt, Germany) was measured only in cold water at the inlet of hotel water systems.

2.5. Statistical analysis

Statistical association between *Legionella* presence (with culture method and Real-Time PCR) and physicochemical parameters were evaluated with χ^2 test.

3. Results

The results of *Legionella* monitoring of water samples are given in **Table 1**.

3.1. Quantitative determination of *Legionella pneumophila* by culture method

As observed in **Table 1** a total of 19 water samples (32%) were contaminated by *L. pneumophila*. In the most of the contaminated hotels (63%) all the 3 sampling sites of hot waters (boiler, bathroom outlets, water recycling) were positive for *L. pneumophila*, while for the other hotels (37%) only one or two of these sites were positive.

L. pneumophila is the only species isolated in the hotel water system monitored. Among the positive hotels the 75% was colonised by *L. pneumophila* serogroups 2 to 14, while only one was colonised by *L. pneumophila* serogroup 1. Mixed *L. pneumophila* cultures (serogroup 1 and serogroups 2 to 14) were obtained in a sole hotel in to different sampling sites.

The *Legionella* contamination for positive samples ranged from 2.0×10^2 to 3.7×10^4 CFU/L. The 58% of positive samples contained $>10^3$ CFU/L and the 21% showed a contamination $>10^4$ CFU/L. In all the hotels that showed a high *Legionella* concentration ($>10^3$ UFC/L) the *L. pneumophila* serogroups 2 to 14 was recovered, while *L. pneumophila* serogroup 1 was isolated only in 3 water samples at lower contamination (range between 10^2 and 10^3 CFU/L).

In the **Figure 2** the percentage of positive samples revealed by the two analytical methods in the different sampling points of hotel water distribution systems is reported. No *Legionella* contamination (< 100 CFU/L) was observed in water samples collected at the inlet of the hotel water system. The hot water recycling resulted the most frequently contaminated site (42%), while the 32% of bathroom outlets and the 26% of boilers were *Legionella* colonised. Even though the hot water recycling was the most colonised site the highest *L. pneumophila* concentration was observed in some bathroom outlets.

3.2. Quantitative determination of *Legionella pneumophila* by Real-Time PCR

The Real-Time PCR revealed the *L. pneumophila* presence in 37 water samples (63%) (**Table 1**). In the 71% of the contaminated hotels all the three sampling sites showed the presence of *L. pneumophila*. In the remaining 29% only one or two sampling sites were contaminated.

The amount of *L. pneumophila* (GU) ranged from 9.3×10 to 5.7×10^4 GU/L. It is important to note that the 15% of the analysed water samples showed a concentration of *L. pneumophila* under the quantification limit (<480 UG/L). In general a low level of Genomic Units was reported in hotels that showed also a low level of contamination with the culture method.

Respect to the culture method the bathroom outlets resulted the more frequently contaminated sampling site using RT-PCR (**Figure 2)**.

This site also showed the highest level of *L. pneumophila* contamination. As observed with the culture method the presence of *L. pneumophila* was not revealed (< 80 GU/L) in water samples collected at the inlet of hotel water system.

3.3. Comparative analysis of water samples by culture method and Real-Time PCR

In **Table 2** the results of the comparative analysis of culture method and Real-Time PCR are reported. The 25% of the samples analysed resulted positive (19/75) by culture method and Real-Time PCR, and the 51% were negative (39/75) by both the assays; otherwise the 24% of the culturally negative samples resulted positive with the Real-Time PCR and any water sample negative with the molecular method was revealed as positive by the culture method. Making a comparison of the level of contamination in the 12 samples positive with both the methods the number of genomic units revealed with Real-Time PCR were higher than the number of CFU observed with the culture method. A linear regression was performed on all hot water samples collected in the distribution systems to compare culture and Real-Time PCR (**Figure 3**). The correlation analysis did not reveal any significant relationship between the two methods (r^2=0.394).

3.4. Physical and chemical analyses

The temperature of the hot water monitored samples ranged between 41.1°C and 70.0 °C **(Table 1)**. As expected, in almost all the investigated hotels (84%) the highest temperature values were found at boiler level (range of 43-70°C). The low temperature values were observed in the bathroom outlets, with a range of 43-53°C in the 86% of cases and these water samples resulted also the most contaminated by *L. pneumophila*.

In the 47% of the monitored hotel (9/19) the water hardness value measured in the cold water entering the distribution system decreases in the boiler and water recycling sites for the presence of softening devices.

The level of free chlorine measured in the inlet water ranged between 0.01 mg/L and 0.08 mg/L showing very low concentrations in comparison with the values recommended in the Italian legislation for drinking water (0.2 mg/L, DLgs, 2001). Considering the low chlorine levels observed, no statistical evaluation was performed to study the influence of the free chlorine concentration and *Legionella* presence in the water.

4. Discussion

Italy is positioned in the first place among European Country for the number of cases of travel-associated Legionellaires' disease. These cases have been often associated to the stay in accommodation sites (Ricketts et al., 2008). Different studies showed that large buildings, mainly the oldest, as the hotels investigated in this work, provided a more hospitable environment than small facilities because the more extensive piping supplied a large surface with variable temperature and biofilm accumulation, factors than favoured the growth and proliferation of *Legionella* spp. (Borella et al., 2005; Yu, 2002).

This study showed that a high percentage of the investigated buildings was colonised by *L. pneumophila* confirming the important role of the hot water distribution system in *Legionella* spreading. In fact the 42% of hotels and 32% of water samples analysed were positive for *L. pneumophila* by culture method. These percentage is lower than that reported in other studies conducted in Italian accommodation sites (75% of hotels and 60-63% of water samples) (Borella et al., 2005; Leoni et al., 2005), but this difference could be due to the different number of water samples analysed in these studies.

The classical culture method allowed to reveal that the 58% of positive samples exceeded 10^3

CFU/L that is the threshold for considering preventive measures according to Italian guidelines (Gazzetta Ufficiale, 2000). The 21% of the examined water samples exceeded 10^4 CFU/L that is the threshold to perform decontamination measures and/or reclamation according to Italian guidelines. Different studies showed that values of *Legionella* contamination higher than 10^4 CFU/L can be considered at high risk for Legionellaires' disease (Rota et al., 2004).

It is important to highlight that all the isolates colonies in this study were *L. pneumophila*. In particular the serogroups 2 to 14, generally associated with the 15-20% of community-acquired cases of Legionellaires' disease, were the most widespread serogroups in the investigated hotels, while *L. pneumophila* serogroup 1, associated with the 80-90% of legionellosis cases, was found only in three hotels at low concentration.

Results showed that, in general, the bathroom outlets located in the rooms near the end of the water distribution system were the most contaminated sampling sites. As reported in other studies (Leoni et al., 2005) this finding may be related to the complexity of the water distribution systems in which the intermittent use of the hot water and the presence of dead-ends can promote water stagnation. Moreover some studies reported that the water samples collected in the bathroom taken from the showers generally were more contaminated than the tap water (Legnani et al., 2002).

As expected, the role of the distribution water system in the colonisation of *L. pneumophila* was highlighted by the lack of finding of this microorganism at the inlet of the hotel water system.

The results of this study underlay that the *Legionella* contamination was significantly influenced ($P < 0.01$, χ^2 test) by water temperature. In particular with the culture method a greater percentage of *Legionella* positive samples was obtained in the water samples with temperature ≤ 55°C (**Tabella 3)**. In particular the percentage of positive samples by culture method were higher in samples that showed temperature ≤ 55°C (**Tabella 3)**. This trend, in agreement with that observed by other authors (Borella et al., 2005; Lasheras et al., 2006; Legnani et al., 2002; Leoni et al., 2005; Mouchtouri et al., 2007), confirmed the importance to maintain the distribution system temperature at values >55°C to avoid legionellae colonisation and proliferation. The *Legionella* contamination

detected by Real-Time PCR was significantly related ($P < 0.01$) to water sample temperature ≤ 60°C. In comparison with the results obtained with the culture method this finding suggests the need to increase the temperature at value > 60°C to guarantee the protection of water system from *Legionella* colonisation. However is important to highlight the ratio between risks and advantages since maintain the water temperature at values > 60°C has different drawbacks as the increased heating cost and the burn risk.

In this study no relationship was found between *Legionella* contamination and water hardness although in other studies the low hardness was recognised as a risk factor for *Legionella* colonisation (Borella et al., 2005; Leoni et al., 2005). Moreover in this study, in contrast with that observed by other authors that found a correlation between low level of hardness and *L. pneumophila* serogroups 1 (Borella et al., 2005), the water hardness did not influence the distribution of the different *L. pneumophila* serogroups.

This results confirmed that quantitative PCR was more sensitive than conventional culture for *L. pneumophila* detection as observed in others works (Aurell et al., 2004; Behets et al., 2007; Declerck et al, 2006; Fiume et al., 2005; Palmer et al. 1993; Wellinghausen et al., 2001; Yaradou et al., 2007). In fact the 24% of Real-Time PCR positive samples were negative by culture method, whereas the opposite situation was never found.

Quantitative real-time PCR gives the number of genomic unit per liter, but a clear equivalence with the number of CFU has not been established, even if different studies compared the results of qPCR and culture in the enumeration of *L. pneumophila* (Joly et al., 2006; Wellinghausen et al., 2001; Yaradou et al., 2007). In this work the number of genomic units measured was higher than the number of CFU detected in the 63% of water samples. This result supports previous finding indicating that culture method frequently underestimates the presence of *L. pneumophila* in water samples (Behets et al., 2007; Levi et al., 2003;). The difference between quantitative values of culture and molecular methods was highlighted by a not significant correlation between the results obtained with the two methods. These observations confirm the ones found in other studies (Joly et

al., 2006; Levi et al., 2003; Morio et al., 2008; Wellinghausen et al., 2001). It is important to note that the presence or absence of a significant correlation between cultural and molecular method can depend largely on the type of water sample analysed (e.g. hot water or cooling tower water as reported (Yaradou et al., 2007). Several factors could contribute to the observed discrepancies between the two methods. In particular Real-Time PCR detects total cell counts including living and dead cells, while culture method only detects living cells that are able to multiply on selective medium (Aurell et al., 2004). Moreover qPCR can detect VBNC legionellae often present in environmental samples. The role of dead and VBNC legionellae in the PCR positive samples was recently demonstrated in a study conducted by Dussere et al. (2008). The Real-Time PCR can also detect the legionellae present in protozoa such as amoebae, that represents an important ecological niche for *Legionella* persistence. Some studies have revealed that filtration of large water samples volumes can cause a loss of up to 90% of culturable bacteria resulting in dead bacteria with preserved DNA only detectable through Real-Time PCR (Levi et al., 2003). Another factor influencing *L. pneumophila* growth on culture medium is the inhibition due to the presence of other microrganisms (Bej et al., 1991).

5. Conclusions

In conclusion the results obtained in this study highlight that the monitored Italian hotels represent a possible source of risk for legionellaires' disease, although almost all the isolated colonies were identified as *L. pneumophila* serogroups 2 to14 and not as *L. pneumophila* serogroup 1.

Referring to the molecular method the real-time PCR proved to be sensitive to detect lower level of contamination in comparison with the culture method. This makes it a promising method that can complement the current culture based standard method for *L. pneumophila* detection in water samples. The Real Time PCR could be particularly useful when outbreak of Legionnaires' disease occurs for the high sensitivity of this assay and for the capability to screen a significant number of

samples in a short time. Moreover this method, for its characteristics, can be used for the preventive screening of water samples complemented by the conventional culture method. The first important limitation for the Real Time-PCR detection techniques was the occurrence of PCR inhibitors in environmental water samples that can lead to false-negative results. For these reasons the use of an internal positive control in PCR is very important to monitor the efficiency of the reaction. The second important limitation is that the large amounts of legionellae detected by PCR may also represent non viable cells or only *Legionella* DNA which is not infectious to human. Therefore, the PCR positive results should be critically interpreted and do not necessarily represent a health risk for exposed persons.

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Figure1. Distribution of the hotels monitored in Italy

Figure2

Figure 2 Percentage of positive samples for *L. pneumophila* contamination in the different sampling points of the hotel water distribution system.

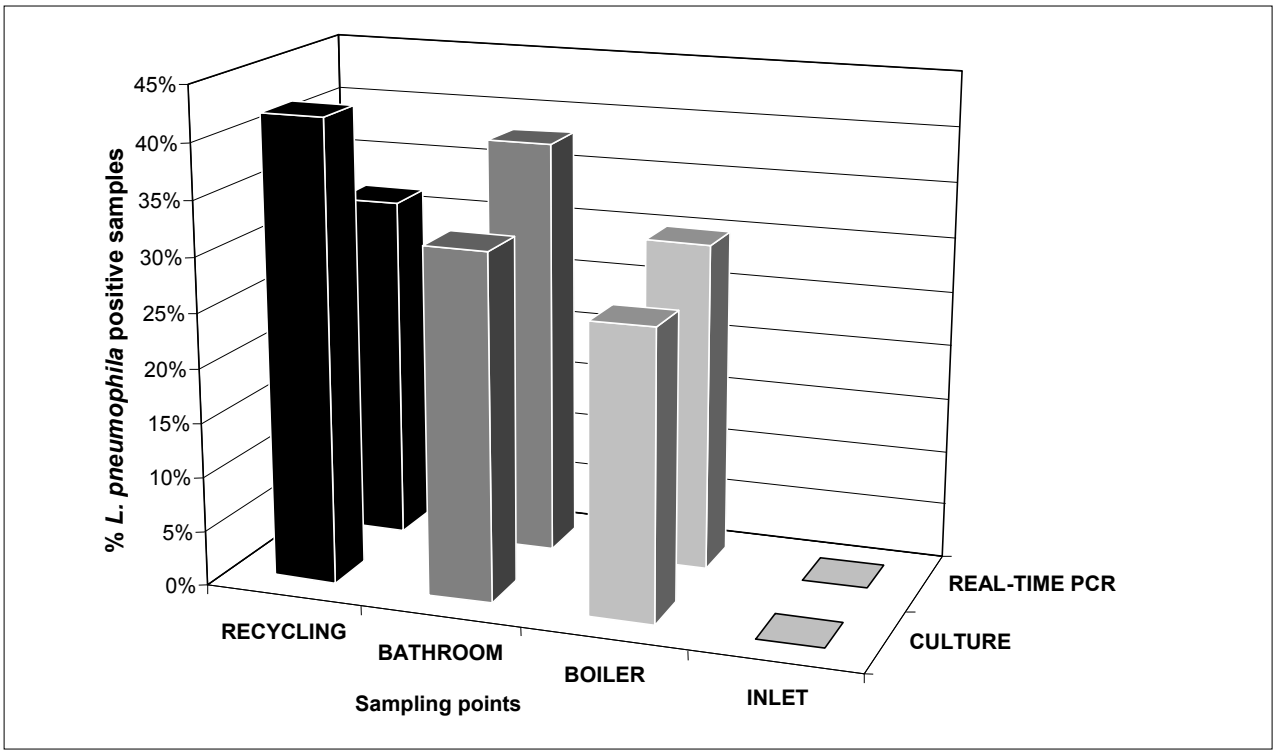

Figure 3. Comparison of culture method results with the Real-Time PCR results. Culture method detection limit 2.00 log (100CFU/L); RT-PCR detection limit 1.9 log (80GU/L).

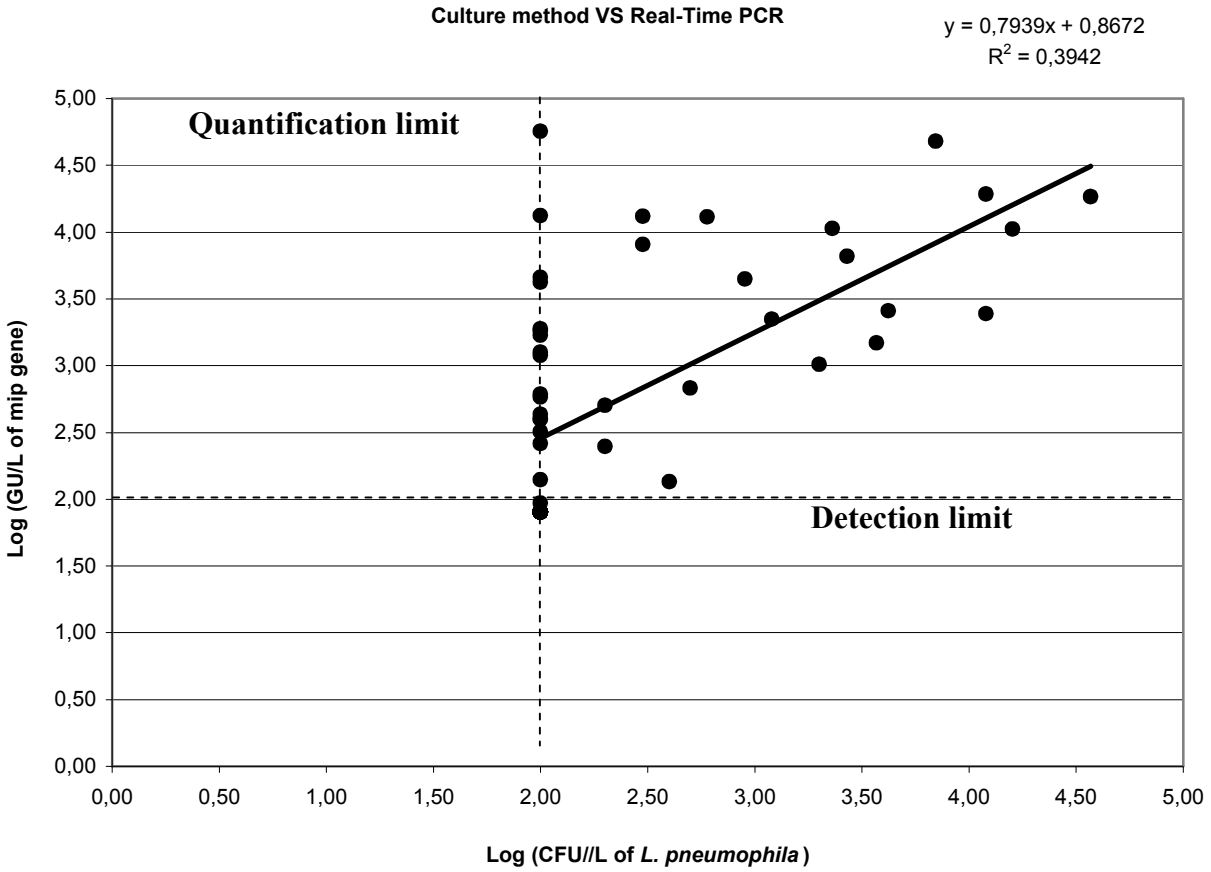

Table1

Table 1. *L. pneumophila* contamination and physico-chemical characteristics of water examined in different hotel sampling sites and cities.

Site	Sampling point	Culture (CUF/L)	Serogroup	Real-time PCR (GU/L)	Temperature (°C)	Free chlorine (mg/L)	Total hardness (°F)
Trieste (TS)	boiler	< 100 [a]	-	1264	66.0	-	6.3
	recycling	< 100	-	4224	55.7	-	8.0
	bathroom	< 100	-	4576	55.3	-	8.0
	inlet	< 100	-	< 80 [b]	-	0.03	18.5
Vicenza (VI)	boiler	< 100	-	< 80	61.0	-	17.5
	recycling	< 100	-	< 80	59.8	-	17.6
	bathroom	< 100	-	< 80	61.2	-	17.6
	bathroom	< 100	-	432 [c]	65.8	-	17.6
Ravenna (RA)	boiler	< 100	-	13280	58,8	-	10.1
	recycling	12000	2-14	2448	52.0	-	10.1
	bathroom	4200	2-14	2576	50.8	-	10.0
	bathroom	< 100	-	56960	41.1	-	9.9
Ancona (AN)	boiler	200	2-14	506	48.3	-	27.5
	recycling	500	2-14	680	34.0	-	27.2
	bathroom	3700	2-14	1482	47.7	-	27.2
	inlet	< 100	-	< 80	-	0.03	26.0
Florence (FI)	boiler	< 100	-	1197	59.0	-	20.6
	recycling	< 100	-	397 [c]	49.8	-	20.7
	bathroom	< 100	-	397 [c]	47.6	-	20.5
	inlet	< 100	-	< 80	-	0.03	20.6
Siena (SI)	boiler	< 100	-	1894	55.6	-	3.0
	recycling	< 100	-	320 [c]	52.2	-	3.5
	bathroom	< 100	-	< 80	50.9	-	3.3
	inlet	< 100	-	< 80	-	0.01	3.8
Pisa (PI)	boiler	< 100	-	93 [c]	70.0	-	8.9
	recycling	200	1	249 [c]	44.3	-	8.5
	bathroom	< 100	-	582	40.2	-	8.8
	inlet	< 100	-	< 80	-	0.01	28.6
Milan	boiler	< 100	-	1696	62.5	-	11.2

(MI 1)	recycling	400	2-14	135 [c]	45.5	-	11.3
	bathroom	< 100	-	611	48.7	-	11.7
	inlet	< 100	-	< 80	-	0.05	30.4
Milan	boiler	< 100	-	< 80	56.3	-	29.8
(MI 2)	recycling	< 100	-	< 80	54.0	-	31.8
	bathroom	< 100	-	1.824	53.4	-	30.2
	inlet	< 100	-	< 80	-	0.01	29.2
Turin	boiler	< 100	-	< 80	42.8	-	12.1
(TO)	recycling	< 100	-	< 80	41.8	-	12.4
	bathroom	< 100	-	< 80	43.0	-	11.5
	inlet	< 100	-	< 80	-	0.05	26.8
Rome	boiler	< 100	-	< 80	60.4	-	8.8
(RM)	recycling	< 100	-	140 [c]	52.6	-	8.6
	bathroom	< 100	-	261 [c]	53.3	-	8.4
	inlet	< 100	-	< 80	-	0.00	6.7
Ischia	boiler	16000	2-14	10560	55.5	-	3.0
(NA 1)	recycling	7000	1	48064	49.6	-	3.0
	bathroom	300	1	13184	48.9	-	2.8
	inlet	< 100	-	< 80	-	0.06	37.8
Naples	boiler	1200	2-14	2227	49.6	-	7.5
(NA 2)	recycling	2300	2-14	10688	48.0	-	7.6
	bathroom	900	2-14	4448	39.7	-	7.4
	inlet	< 100	-	< 80	-	0.08	7.9
Bologna	boiler	300	2-14	8096	56.2	-	2.2
(BO)	recycling	600	2-14	12992	50.2	-	2.2
	bathroom	37000	2-14	18400	53.3	-	2.1
	inlet	< 100	-	< 80	-	0.025	35.7
Lecco	boiler	< 100	-	< 80	55.4	-	8.4
(LC)	recycling	< 100	-	< 80	42.8	-	8.6
	bathroom	< 100	-	< 80	50.1	-	8.2
	inlet	< 100	-	< 80	-	0.01	22.5
Bergamo	boiler	< 100	-	< 80	67.2	-	6.7
(BG)	recycling	< 100	-	< 80	48.9	-	6.8

	bathroom	< 100	-	< 80	47.8	-	6.7
	inlet	< 100	-	< 80	-	0.01	17.8
Genoa (GE)	boiler	< 100	-	< 80	54.3	-	14.0
	recycling	< 100	-	< 80	57.4	-	13.6
	bathroom	< 100	-	< 80	59.4	-	13.7
	inlet	< 100	-	< 80	-	0.00	12.1
La Spezia (SP)	boiler	2000	2-14	1024	53.0	-	24.0
	recycling	2700	2-14	6592	43.2	-	23.2
	bathroom	12000	2-14	19232	42.9	-	23.4
	inlet	< 100	-	< 80	-	0.00	23.9
Brescia (BS)	boiler	< 100	-	< 80	62.5	-	27.2
	recycling	< 100	-	< 80	59.5	-	27.2
	bathroom	< 100	-	< 80	46.1	-	28.6
	inlet	< 100	-	< 80	-	0.00	32.9

[a] < 100 CFU/L: detection limit (culture method)

[b] < 80 GU/L: detection limit (Real-Time PCR)

[c] < 480 GU/L: quantification limit (Real-Time PCR)

Table 2 Comparative detection of *Legionella pneumophila* in water samples by culture method and Real-Time PCR

	Real-Time PCR +	Real-Time PCR -	Total
Culture +	19 (25%)	0 (0%)	19 (25%)
Culture -	18 (24%)	39 (51%)	57 (75%)
Total	37 (49%)	39 (51%)	76 (100%)

Table3

Tabella 3. Relationship between positive samples for *L. pneumophila* contamination and physico-chemical parameters.

	Temperature (°C)		Total hardness (°F)	
	≤ 55	> 55	≤ 15	> 15
Culture +	29% [a]	3%	22%	10%
Culture -	34%	34%	46%	22%
	≤ 60	> 60	≤ 15	> 15
Real-Time PCR +	56% [a]	7%	44%	19%
Real-Time PCR -	29%	8%	24%	13%

[a]: $P < 0.01$, according to χ^2 test