Original Article

Assessment of *Legionella pneumophila* in recreational spring water with quantitative PCR (Taqman) assay

Shu-Min Shen[1], Ming-Yuan Chou[2]*, Bing-Mu Hsu[1], Wen-Tsai Ji[1]*, Tsui-Kang Hsu[3]*, Hsiu-Feng Tsai[1], Yu-Li Huang[4]*, Yi-Chou Chiu[5], Erl-Shyh Kao[6], Po-Min Kao[1], Cheng-Wei Fan[1]

[1]Department of Earth and Environmental Sciences, National Chung Cheng University, Chiayi, Taiwan, Republic of China, [2]Department of Internal Medicine, Cheng Hsin General Hospital, Taipei, Taiwan, Republic of China, [3]Department of Ophthalmology, Cheng Hsin General Hospital, Taipei, Taiwan, Republic of China, [4]Department of Safety Health and Environmental Engineering, National Kaohsiung First University of Science and Technology, Kaohsiung, Taiwan, Republic of China, [5]General Surgery, Surgical Department, Cheng Hsin General Hospital, Taipei, Taiwan, Republic of China, [6]Department of Beauty Science and Graduate Institute of Beauty Science Technology, Chienkuo Technology University, Changhua, Taiwan, Republic of China

Legionella spp. are common in various natural and man-made aquatic environments. Recreational hot spring is frequently reported as an infection hotspot because of various factors such as temperature and humidity. Although polymerase chain reaction (PCR) had been used for detecting *Legionella*, several inhibitors such as humic substances, calcium, and melanin in the recreational spring water may interfere with the reaction thus resulting in risk underestimation. The purpose of this study was to compare the efficiencies of conventional and Taqman quantitative PCR (qPCR) on detecting *Legionella pneumophila* in spring facilities and in receiving water. In the results, Taqman PCR had much better efficiency on specifying the pathogen in both river and spring samples. *L. pneumophila* was detected in all of the 27 river water samples and 45 of the 48 hot spring water samples. The estimated *L. pneumophela* concentrations ranged between 1.0×10^2 and 3.3×10^5 cells/l in river water and 72.1–5.7×10^6 cells/l in hot spring water. Total coliforms and turbidity were significantly correlated with concentrations of L. pneumophila in positive water samples. Significant difference was also found in water temperature between the presence/absence of *L. pneumophila*. Our results suggest that conventional PCR may be not enough for detecting *L. pneumophila* particularly in the aquatic environments full of reaction inhibitors.

Keywords: *L. pneumophila*, Quantitative PCR, Hot spring, River

Introduction

Legionella spp. was first isolated from guinea pigs in 1934, but its potential hazards were not recognized until the first large-scale pneumonia outbreak in Philadelphia, USA in 1976.[1,2] At least 59 *Legionella* species have been identified since, and 26 species are associated with human illness, including Legionnaires' disease and Pontiac fever.[3] *Legionella pneumophila* is the most common pathogenic *Legionella* species, accounting for 80–90% of Legionnaires' disease cases.[4,5]

Legionella spp. can be found in natural and man-made water environments such as rivers, hot springs, lakes, cooling towers, and swimming pools. Until recently, many of the *Legionella* monitoring studies were performed in response to legionellosis outbreaks.[6–8] According to previous studies, the detection rate of *Legionella* spp. ranged between 25.8 and 64% in man-made water facilities,[9–12] and the concentration varied from 940 to 1.5×10^6 genome units (GU)/l.[12,13] For natural water environment, the detection rate of *Legionella* spp. was 8.7–100%, and concentrations ranged between 7.4×10^3 and 9.4×10^5 GU/l,[14] and 10^4–10^8 cells/l.[15,16]

Polymerase chain reaction assay has been widely used for detection of microbes in environmental samples. Compared to conventional incubation method, PCR is less time-consuming and more

Correspondence to: Bing-Mu Hsu, Department of Earth and Environmental Sciences, National Chung Cheng University, 168, University Road, Min-Hsiung, Chiayi, Taiwan, Republic of China. Email: bmhsu@ccu.edu.tw

*Equal contribution to first author.

DOI 10.1179/2047773215Y.0000000023

sensitive in detecting and identifying specific microorganisms. Although highly specific, PCR is more vulnerable to aquatic pollutants since concentration of bacteria by membrane filtration usually comes with residues of miscellaneous PCR inhibitors including humic substances, metal ions, polysaccharides, and insoluble debris.[17–21] Such inhibitors interfere with PCR via inactivating DNA polymerase or sequestering/degrading DNA templates.[21] In the recreational spring water, higher concentration of calcium and the impurities such as the melanin from hair and skin, and the urea from human discharges are all potential PCR inhibitors.[22] Hot spring facility is a known hotspot of *Legionella* infection owing to the warmer temperature and humidity, which provides an ideal environment for the bacterial growth. Water swirling and shower nozzles may further increase the pathogens in aerosol which could further increase the infection possibility. However, PCR inhibitors in the recreational spring water may result in underestimation of the *Legionella* risk.

In this study, qPCR (Taqman) was used for detecting and quantifying *L. pneumophila* in hot spring and river samples. The efficiencies between the conventional PCR and Taqman qPCR were compared. In addition, relationship between the presence of *L. pneumophila* and various water quality parameters were evaluated.

Materials and Methods

Sampling sites, samples collection, and water quality analysis

Water samples were collected from along Puzih River (23°28′N, 120°13′E) in southern Taiwan and from three hot spring recreational areas. The three hot spring areas included K.-K. (24°20′N, 121°01′E), P.-L. (22°41′N, 121°00′E), and Z.-P. (22°05′N, 120°44′E), and all are with weak alkaline-carbonated hot springs. The sampling campaign was carried out between August 2011 and July 2012. For each sample, 2,000 ml of water was collected into two sterile 1 lbottles and stored at 4°C before transferring to the laboratory. Three water quality parameters were measured for each water sample at the time of sample collection, including pH level with a portable pH meter (D-24E, Horiba Co., Japan), water temperature with a thermometer, and turbidity with a turbidimeter (HACH Co., Loveland, CO). In addition, microbial water quality was assessed within 24 hours of sample collection, including heterotrophic bacteria by spread method, and total coliforms by membrane filtration and incubation with a differential medium as prescribed in the Standard Method for the Examination of Water and Wastewater (Methods 9215 C and 9222 B).[23] Statistical analyses (differences and correlation) on presence and amount of *L. pneumophila* with the water quality parameters were performed using the STATISTICA® software (StatSoft, Inc., Tulsa, USA).

Sample pretreatment

One liter of each water sample was filtered through 0.22 μm pore size, 47-mm diameter cellulose nitrate membranes (Pall, New York, USA). The number of membranes used for each sample depended on the water turbidity. After filtration, the membranes were collected and swirled with 100 ml of Page's saline solution for 5 min to elute the microbes from the membranes for each sample. Page's saline solution has prepare in 1 l of de-ionized water contained 120 mg of NaCl, 4 mg of $MgSO_4.7H_2O$, 4 mg of $CaCl_2.6H_2O$, 142 mg of Na_2HPO_4, and 136 mg of KH_2PO_4. The resulting eluent was transferred into two conical centrifuge tubes (50 ml each) and centrifuged at 5,800 × *g* for 30 min. For each centrifuged solution, the top supernatant fluid (about 47.5 ml) was aspirated and discarded. The pellet in the remaining 2.5 ml solution was resuspended by vortexing in a disinfected tube. For each water sample, two tubes of 2.5 ml concentrate were produced. DNA extraction was done with MagNA Pure LC System (Roche, Basel, Switzerland) and MagNA Pure LC DNA Isolation Kit III (Roche). The derived DNA was used for all the PCRs.

Detection and quantification of L. pneumophila with qPCR (Taqman) assay

In this study, *mip* was used as the target gene for identifying *L. pneumophila*. The operation conditions of qPCR for *L. pneumophila* are summarized in Table 1. The qPCR mixtures contained 5 μl template DNA, 0.5 μl of each primer, 0.5 μl of probe, 10 μl of probe mix, and sterile water to 20 ml volume. After qPCR, the reaction product was examined for the presence of specific target genes using electrophoresis separation. Nuclease-free water (Qiagen) was used in all experiments as negative controls. Each qPCR run was conducted using positive control DNA for *L. pneumophila* ATCC 33823, sample DNA, and negative control.

Calibration for quantification outcome by qPCR (Taqman) assay

The positive control, *L. pneumophila* ATCC 33823, was transferred onto a BCYE agar plate (buffered charcoal yeast extract with alpha-ketoglutarate, L-cysteine, and ferric pyrophosphate). The inoculated plates were then incubated in a 5% CO_2 incubator at 37°C. DNA was extracted from a 5-day culture of *L. pneumophila* by heating method (heating at 95°C for 10 min, followed by centrifuge at 9,700 × *g* for 10 min) for qPCR (Taqman) assay. The qPCR products were identified with gel electrophoresis on a 2% agarose gel (Biobasic Inc., New York, USA) with 5 μl reaction solution from qPCR (Taqman) assay. The band of gel in target gene site (66 bp) was cut carefully before gel dissociation. The DNA

Table 1 Summary of primer sequences of polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays for *Legionella* spp. and *L. pneumophila*.

PCR assay	Primer sequences		Predenaturation, denaturation, annealing and extension temperature			Cycling Number	Amplicon size (bp)	Reference
PCR	*leg* 225 5′-AAGATTAGCCTGCGTCCGAT-3′ *leg* 858 5′-GTCAACTTATCGCGTTTGCT-3′	–	95	62	72	40	654	Miyamoto *et al.*[24]
	mip 920 5′-GCTACAGACAAGGATAAGTTG-3′ *mip* 1548 5′-GTTTTGTATGACTTTAATTCA-3′	–	95	62	72	40	648	Bej *et al.*[25]
*q*PCR	*mip* F 5′-TTCATTTGYTGYTCGGTTAAAGC-3′ *mip* R 5′-AWTGGCTAAAGGCATGCAAGAC-3′ probe 5′(FAM)-AGCGCCACTCATAG-(BHQ1) 3′	95	95	60	72	40	66	Behets *et al.*[26]

leg: Legionella eukaryotic-like genes; *leg* 225/858 is specific for *Legionella* species; *mip*: macrophage infectivity potentiator; *mip* 920/1548 and *mip* F/R is specific for *Legionella pneumophila*.

was purified by Gel/PCR DNA fragments extraction kit (Geneaid, New Taipei City, Taiwan). Subsequently, yT&A clone vector kit (Yeasterm Biotech Corporation, Taipei, Taiwan) was used and the vector will ligate with the insert DNA after overnight reaction at 4°C. The transformed cells were incubated overnight at 37°C for blue/white screening, with white colonies chosen after competent cell transformation. The recombinant plasmid DNA was purified by HiYield™ plasmid mini kit (Real Biotech Corporation, Taipei, Taiwan), and the plasmid DNA amount was determined by Micro-spectrophotometer (CLUBIO CB-4500, Taiwan) at 260 nm in triplicates. The corresponding copy number was calculated using the following equation[27]

$$\text{DNA (copy number)} = \frac{6.02 \times 10^{23}(\text{copy/mol}) \times \text{plasmid DNA amount(g)}}{\text{DNA length (bp)} \times 660\,(\text{g/mol/dp})}$$

Quantification of *L. pneumophila* was determined with threshold cycle value (Ct) by which the threshold fluorescence level was detected by the ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Singapore). A detected *L. pneumophila* cell would be counted as one copy number,[28] thus the calculated Ct may be converted to *L. pneumophila* concentration in cells/l. Plasmid DNA of *L. pneumophila* from positive control was used with serial 10-fold dilutions by sterile water for qPCR(Taqman) assay, and the resulting copy numbers were compared with observed Ct to construct the calibration curve for quantifying *L. pneumophila* in the water sample.

PCR analysis

In this study, the PCR method was used for comparison with detection outcomes by qPCR. The PCR solution was prepared with 5 µl of the DNA templates and PCR mixture to a total volume of 50 µl. The PCR mixture contained 5 µl 10× PCR buffer (20 mM $MgCl_2$), 1 µl dNTP mix (10 mM of each dNTP), 200 pmol each of the oligonucleotide primers and 0.3 µl VioTaqTM DNA Polymerase (Viogene, New Taipei City, Taiwan, 5 U/µl), and DNase-free deionized water. The primer sets and reaction settings for *Legionella* spp. and *L. pneumophila* are summarized in Table 1. The target genes (*leg* and *mip*) were used to confirm the detection of *L. pneumophila.* The PCR product was confirmed for the presence of specific target genes using electrophoresis separation. *L. pneumophila* ATCC 33823 was used as positive control in this study, and negative controls were also included by replacing the DNA template with distilled water for subsequent analyses.

Gel electrophoresis

All positive samples, whether detected by qPCR or PCR method, were subject to gel electrophoresis for species confirmation on a 2% agarose gel (Biobasic Inc.) with 5 µl reaction solution. The DNA fragments were confirmed with ethidium bromide staining (0.5 µg/ml, 10 min). A 100-bp DNA ladder was used as a DNA size marker for image production under UV light.

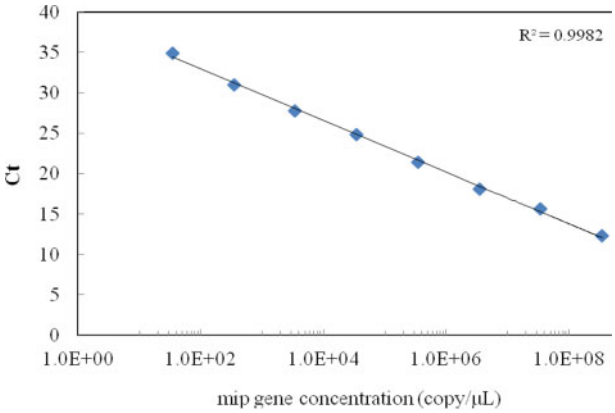

Figure 1 The calibration curve of quantitative *L. pneumophila mip* polymerase chain reaction (PCR).

Table 2 ***L. pneumophila*** **monitoring for molecular techniques in various types of water samples.**

Type of water sample	Detection rate (%)*			Range of *L. pneumophila* concentration (cells/l, average concentration)+
	leg PCR	*mip* PCR	*mip* qPCR	
Overall	54.7 (41/75)	26.7 (19/75)	96.0 (72/75)	$72.1–5.7 \times 10^6$ (1.5×10^4)
Puzih river	70.4 (19/27)	7.4 (2/27)	100.0 (27/27)	$1.0 \times 10^2–3.3 \times 10^5$ (1.2×10^4)
Hot spring	45.8 (22/48)	35.4 (17/48)	93.8 (45/48)	$72.1–5.7 \times 10^6$ (2.5×10^4)
Source water	58.3 (7/12)	41.7 (5/12)	100.0 (12/12)	$1.7 \times 10^2–5.7 \times 10^6$ (8.4×10^5)
Hot spring water	50.0 (5/10)	40.0 (4/10)	100.0 (10/10)	$1.7 \times 10^2–5.7 \times 10^6$ (1.0×10^6)
Cold water	100.0 (2/2)	50.0 (1/2)	100.0 (2/2)	$3.6 \times 10^2–8.0 \times 10^2$ (5.8×10^2)
Facility water	42.4 (14/33)	33.3 (11/33)	90.9 (30/33)	$72.1–6.5 \times 10^5$(4.1×10^4)
Public hot tub	38.9 (7/18)	33.3 (6/18)	94.4 (17/18)	$72.1–6.5 \times 10^5$ (6.1×10^4)
Spa	66.7 (6/9)	55.6 (5/9)	88.9 (8/9)	$1.4 \times 10^2–1.3 \times 10^5$ (1.7×10^4)
Personal hot tub	16.7 (1/6)	0.0 (0/6)	83.3 (5/6)	$1.6 \times 10^2–6.6 \times 10^4$ (1.4×10^4)
Wastewater	33.3 (1/3)	33.3 (1/3)	100.0 (3/3)	$2.3 \times 10^3–3.6 \times 10^5$ (1.2×10^5)

*Numbers in parentheses indicate positive and total samples, respectively. +Quantitative assessment outcomes from positive samples only.

Results and Discussion

Calibration outcome for qPCR (Taqman) assay

The calibration outcome for qPCR (Taqman) assay is presented in Fig. 1. A linear relationship was observed between Ct values and the logarithmic values of the *mip* gene copy numbers, and the calibration equation was $Ct = -1.39 \log_{10}$ [copy number] $+ 39.33$. The coefficient of determination (r^2) was 0.998, with range of detection from 34.3 to 3.5×10^8 cells/l. The calibration results showed that this method would allow a wide range for *L. pneumophila* concentration.

L. pneumophila monitoring outcomes

A total of 75 water samples were collected from a river and three hot spring recreation areas in this study. Each sample was evaluated with PCR and qPCR, and the detection outcomes are summarized in Table 2. Of the 75 samples analyzed, 72 (96.0%) were found to contain *L. pneumophila* by qPCR. In contrast, 41 positive samples were detected with *leg* PCR and 19 samples with *mip* PCR. As all positive detections were confirmed by electrophoresis, the likelihood of false-positive detection by qPCR was minimized. The estimated concentrations of *L. pneumophila* in the positive samples ranged between 72.1 and 5.7×10^6 cells/l.

Of the 75 water samples, 27 were collected from the same river and 48 were taken from three hot spring recreation areas. The detection rates also varied greatly by type of water source and detection method used (Table 2). With qPCR method, *L. pneumophila* was detected in all (27/27) river samples and 93.8% (45/48) of the samples from hot spring areas. In the results, *L. pneumophila* was detected in all river samples, suggesting that the potential human pathogen may be highly prevalent in the natural environment. Further assessment is warranted with respect to potential infection risks associated with recreational use of the water environment. However, it should not be ruled out that qPCR might overestimate cell counts due to nonspecific signals near the detection limits.[29,30]

In comparison, the detection rates of conventional PCR are consistently lower than qPCR outcomes. Interestingly, the detection rate of *mip* PCR is lower than *leg* PCR especially in the river samples possibly because the primers with higher A/T ratio were more sensitive to PCR inhibitors in receiving waters. On the other hand, evaluation of PCR and TaqMan PCR in artificial samples that inhibitor (Urea) was tested. The results are shown in Fig. 2. TaqMan PCR had much better efficiency on specifying the pathogen in artificial samples. In both Puzih River and hot spring facilities, Taqman qPCR significantly has better efficiency on detecting *L. pneumophila*. These results suggested that Taqman qPCR is less vulnerable to the environmental PCR inhibitors and may be more suitable for evaluation of the pathogens in recreational spring waters.

Quantitative assessment L. pneumophila in hot springs

As shown in Table 2, 45 (93.8%) of the 48 water samples from three hot spring recreation areas were found to contain *L. pneumophila* and the concentrations ranged from 72.1 to 5.7×10^6 cells/l. Within each hot spring recreation area, separate samples

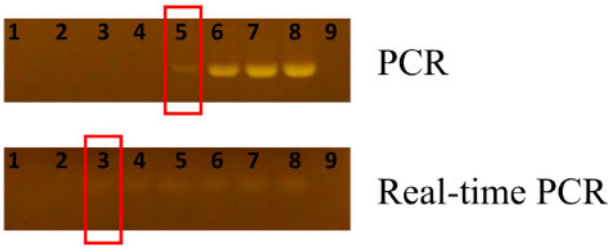

Figure 2 The evaluation of PCR and qPCR inhibitors in artificial samples. DNA concentration: 40 ng/μl, PCR inhibitor: urea. Lane 1:640 mM/μl, lane 2:320 mM/μl, lane 3:160 mM/μl, lane 4:80 mM/μl, lane 5:40 mM/μl, lane 6:20 mM/μl, lane 7:10 mM/μl, lane 8: positive, lane 9: negative.

Table 3 Comparisons of selected water quality parameters between *L. pneumophila* detection outcomes and nonparametric test results

	Mean ± SD		*p*-levels for nonparametric test	
Water quality parameter	*L. pneumophila*-presence ($n = 72$)	*L. pneumophila*-absence ($n = 3$)	Mann–Whitney *U* test	Spearman's rank test ($n = 72$)
Heterotrophic plate counts (HPCs) (CFU/ml)	$1.4 \times 10^5 \pm 3.6 \times 10^5$	$2.1 \times 10^5 \pm 3.0 \times 10^5$	0.612	0.524
Total coliforms (CFU/100 ml)	$5.6 \times 10^3 \pm 9.8 \times 10^3$	44.9 ± 67.5	0.069	0.022*
Turbidity (NTU)	$1.5 \times 10^2 \pm 2.2 \times 10^2$	0.7 ± 0.4	0.231	0.004*
Temperature (°C)	33.3 ± 11.3	47.7 ± 5.1	0.041*	0.116
pH value	7.5 ± 0.7	8.2 ± 1.0	0.201	0.723

* Significant difference at alpha = 0.05

were collected from source water, facilities water, and wastewater. The detection rates ranged from 90.9% in facility water to 100% in source water and wastewater. In addition, the concentrations of *L. pneumophila* also varied widely by sample source, which ranged up to four orders of magnitude in source and facility water samples. The highest concentration was found among source water samples, but the average concentrations were not significantly different by water type, probably due to large variations and small sample size of the source water ($n=12$) and wastewater ($n=3$).

The source waters used in hot spring recreation areas include cool stream water and hot spring water. The average concentration of *L. pneumophila* in positive hot spring samples (1.0×10^6 cells/l) was higher than that in cool stream water (5.8×10^2 cells/l). *Legionella* spp. is known to thrive in hot environment and *L. pneumophila* has been found to grow in pipelines for hot spring waters. It is likely that *L. pneumophila* found in the source water samples may have come from soil or other sources during hot spring collection process (Wallis and Robinson 2005).

Typical facilities in the hot spring recreation areas include public hot tubs, spas, and personal hot tubs. According to qPCR results, the average concentration of *L. pneumophila* in positive samples from public hot tubs (6.1×10^4 cells/l) was slightly higher than those in spas (1.7×10^4 cells/l) and personal hot tubs (1.4×10^4 cells/l). The lower concentrations in water samples from personal hot tubs may have been a result of frequent draining, which prevented proliferation within the facility. For wastewater, only one sample was collected from each hot spring recreational area, and all were found to contain *L. pneumophila*, with concentrations between 2.3×10^3–3.6×10^5 and averaged 1.2×10^5 cells/l.

Relationships between occurrence of L. pneumophila and water quality parameters

Several water quality parameters were measured at each sampling location during sample collection, including heterotrophic plate counts (HPCs), total coliforms, temperature, pH, and turbidity. The average and standard deviation of these water quality parameters are summarized in Table 3. Significant difference was found between the presence and absence of *L. pneumophila* for water temperature ($P=0.041$, Mann–Whitney *U* test). The finding was consistent with that reported by Zanetti.[31] Correlation between concentration of *L. pneumophila* and the five water quality parameters were also evaluated using Spearman's rank-order correlation coefficient test. Significant correlations were found between concentration of L. pneumophila and total coliforms and turbidity. The results in this study were in good agreement with those of our previous report, which indicated that total coliforms in the aquatic environment may be from the soil or fecal contamination.[32] A previous study also suggested that organic contents in sediment might support microbial growth in the aquatic environment, which may in turn increase water turbidity (Valster *et al.*, 2011).

In this study, the qPCR (Taqman) combined with electrophoresis is a rapid and highly sensitive procedure for quantitative assessment of *L. pneumophila* in natural and man-made water environment. The high prevalence and concentration of *L. pneumophila* in hot spring water may pose a significant health risk. Further assessment is necessary to determine potential health risks associated with recreational water contact. To reduce the infection risk, the devices should be completely disinfected regularly and the hot spring could be mixed with chlorinated water for temperature adjustment. Visitors should avoid getting choked with the hot spring water. Frequent drainage and cleaning of the recreational facility were also required to reduce potential health risks.

Disclaimer Statements

Contributors

SMS, MYC, BMH, WTJ, TKH and YLH contributed to the study concept, design, data collection,

analysis, manuscript preparation and approval. PMK, HFT, YCC, ESK and CWF contributed to data collection, analysis, and manuscript preparation and approval.

Funding

This work was supported by research grants from National Science Council of Taiwan, ROC (NSC 100-2116-M-194-004-MY2) and Cheng Hsin General Hospital, Taiwan, ROC (CH104-2).

Conflicts of interest

All authors have agreed to publish the results.

Ethics approval

No ethics concern in this study.

References

1 Tatlock H. A Rickettsia-like organism recovered from guinea pigs. Proc Soc Exp Biol Med. 1944;57:95–9.
2 Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ. Legionnaires' disease: description of an epidemic of pneumonia. N Engl J Med. 1977;297:1189–97.
3 Gomez-Valero L, Rusniok C, Buchrieser C. *Legionella pneumophila*: population genetics, phylogeny and genomics. Infect Genet Evol. 2009;9(5):727–39.
4 Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, Widmer A, *et al.* Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: An international collaborative survey. J Infect Dis. 2002;186(1):127–8.
5 Yanez MA, Nocker A, Soria-Soria E, Murtula R, Martinez L, Catalan V. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J Microbiol Meth. 2011;85(2):124–30.
6 Watson JM, Mitchell E, Gabbay J, Maguire H, Boyle M, Bruce J, *et al.* Piccadilly Circus legionnaires' disease outbreak. J Public Health Med. 1994;16(3):341–7.
7 Joseph CA, Harrison TG, Ilijic-Car D, Bartlett CL. Legionnaires' disease in residents of England and Wales: 1996. Commun Dis Rep CDR Rev. 1997;7(11):R153–9.
8 Marston BJ, Lipman HB, Breiman RF. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. Arch Intern Med. 1994;154(21):2417–22.
9 Hsu BM, Lin CL, Shih FC. Survey of pathogenic free-living amoebae and *Legionella* spp. in mud spring recreation area. Water Res. 2009;43(11):2817–28.
10 Huang SW, Hsu BM, Wu SF, Fan CW, Shih FC, Lin YC, *et al.* Water quality parameters associated with prevalence of Legionella in hot spring facility water bodies. Water Res. 2010; 44(16):4805–11.
11 Huang SW, Hsu BM, Chen NH, Huang CC, Huang KH, Chen JS, *et al.* Isolation and identification of *Legionella* and their host amoebae from weak alkaline carbonate spring water using a culture method combined with PCR. Parasitol Res. 2011;109(5):1233–41.
12 Lee JV, Lai S, Exner M, Lenz J, Gaia V, Casati S, *et al.* An international trial of quantitative PCR for monitoring *Legionella* in artificial water systems. J Appl Microbiol. 2011; 110(4):1032–44.
13 Wery N, Bru-Adan V, Minervini C, Delgenes JP, Garrelly L, Godon JJ. Dynamics of Legionella spp. and bacterial populations during the proliferation of *L. pneumophila* in a cooling tower facility. Appl Environ Microbiol. 2008;74(10):3030–7.
14 Parthuisot N, West NJ, Lebaron P, Baudart J. High diversity and abundance of *Legionella* spp. in a Pristine River and impact of seasonal and anthropogenic effects. Appl Environ Microbiol. 2010;76(24):8201–10.
15 Ortiz-Roque CM, Hazen TC. Abundance and distribution of *Legionellaceae* in Puerto Rican waters. Appl Environ Microbiol. 1987;53(9):2231–6.
16 Declerck P, Behets J, van Hoef V, Ollevier F. Detection of Legionella spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. Water Res. 2007;41(14):3159–67.
17 Rossen L, Norskov P, Holmstrom K, Rasmussen OF. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol. 1992;17(1):37–45.
18 Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and Environmental Microbiology. 1996;62(3):1102–6.
19 Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol. 1997;63(10):3741–51.
20 Burtscher C, Wuertz S. Evaluation of the use of PCR and reverse transcriptase PCR for detection of pathogenic bacteria in biosolids from anaerobic digestors and aerobic composters. Appl Environ Microbiol. 2003;69(8):4618–27.
21 Kermekchiev MB, Kirilova LI, Vail EE, Barnes WM. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. Nucleic Acids Res. 2009;37(5):e40.
22 Radstrom P, Knutsson R, Wolffs P, Lovenklev M, Lofstrom C. Pre-PCR processing – strategies to generate PCR-compatible samples. Mol Biotechnol. 2004;26(2):133–46.
23 APHA. Standard method for the examination of water and wastewater. 21st edn. Washington, DC: APHA, WEF and AWWA; 2005.
24 Miyamoto H, Yamamoto H, Arima K, Fujii J, Maruta K, Izu K, *et al.* Development of a new seminested PCR method for detection of Legionella species and its application to surveillance of Legionellae in hospital cooling tower water. Appl Environ Microb. 1997;63(7):2489–94.
25 Bej AK, Mahbubani MH, Atlas RM. Detection of viable Legionella pneumophila in water by polymerase chain reaction and gene probe methods. Appl Environ Microbiol. 1991;57(2): 597–600.
26 Behets J, Declerck P, Delaedt Y, Creemers B, Ollevier F. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of Legionella pneumophila in water samples. J Microbiol Methods. 2007;68(1):137–44.
27 Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods. 2003;278(1–2):261–9.
28 Koide M, Saito A, Kusano N, Higa F. Detection of Legionella spp. in cooling tower water by the polymerase chain reaction method. Appl Environ Microbiol. 1993;59(6): 1943–6.
29 Rawsthorne H, Dock CN, Jaykus LA. PCR-based method using propidium monoazide to distinguish viable from nonviable bacillus subtilis spores. Applied and environmental microbiology. 2009;75(9):2936–9.
30 Chen NT, Chang CW. Rapid quantification of viable *legionellae* in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J Appl Microbiol. 2010; 109(2):623–34.
31 Zanetti F, Stampi S, De Luca G, Fateh-Moghadam P, Antonietta M, Sabattini B, *et al.* Water characteristics associated with the occurrence of *Legionella pneumophila* in dental units. Eur J Oral Sci. 2000;108(1):22–8.
32 Hsu BM, Chen CH, Wan MT, Cheng HW. *Legionella* prevalence in hot spring recreation areas of Taiwan. Water Res. 2006;40(17):3267–73.