DEVELOPMENT OF A COMBINED AIR SAMPLING AND QUANTITATIVE REAL-TIME PCR METHOD FOR DETECTION OF *LEGIONELLA* SPP

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Abstract. The objective of this study was to develop and optimize the combined methods of air sampling and real time polymerase chain reaction (real-time PCR) for quantifying aerosol *Legionella* spp. Primers and TaqMan hydrolysis probe based on 5S *rRNA* gene specific for *Legionella* spp were used to amplify a specific DNA product of 84 bp. The impinger air sampler plus T-100 sampling pump was used to collect aerosol *Legionella* and as low as 10 fg of *Legionella* DNA per reaction could detected. Preliminary studies demonstrated that the developed method could detect aerosol *Legionella* spp 1.5-185 organisms /500 l of air within 5 hours, in contrast to culture method, that required a minimum of 7-10 days.

INTRODUCTION

Legionella are the causative agents of Legionnaire's disease. Although the genus *Legionella* comprises more than 40 species with 64 serogroups (Benson and Fields, 1998), *L. pneumophila* is the most common pathogenic species, accounting for more than 90% of legionellosis cases (Yu, 2000). *Legionella* are found in moist environment such as cooling towers, evaporative condensers, humidifiers, and wastewater (Heller *et al*, 1998), and may survive as intracellular parasites of amebae and ciliates (Fields *et al*, 2002). The disease is transmitted by inhalation of aerosols containing *Legionella* spp and people with impaired immune systems tend to be most at risk (Roig and Rello, 2003).

The detection of *L. pneumophila* in clinical and environmental samples is an important component of monitoring and control of legionellosis. Nucleic acid amplification techniques, mainly polymerase chain reaction (PCR), are described useful tools for the detection of *L. pneumophila;* however conventional PCR is a qualitative assay, informing only the presence or absence of the microorganism. Recent advent of fluorescent probe-based PCR technology (real-time PCR) has led to the development of quantitative assays. Several studies have used real-time PCR for detection of *Legionella* spp from clinical and water samples and most are based on the amplification of the macrophage infectivity potentiator (MIP) virulent gene (Ballard *et al*, 2000; Templeton *et al,* 2003; Wilson *et al,* 2003), *16s rRNA* gene (Reisch *et al*, 2002), and *Dot A* gene (Yanez *et al*, 2005). Here we describe a novel methodology for the detection and quantification of aerosol *Legionella* spp using quantitative real-time PCR technology.

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MATERIALS AND METHODS

Bacterial strains

Legionella pneumophila serogroup 1 (ATCC 33125) were kindly supplied by the Thai National Institute of Health. The following clinical bacterial strains were kindly supplied by the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, and

used as negative controls: *Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pyrogenes, Streptococcus pneumoniae, Staphylococcus aureus, Acinetobacter* spp, *Enterobacter cloacae, Enterococcus* spp, and *Mycobacterium tuberculosis.*

Aerosol generation

A stock suspension of *L. pneumophila* organisms was prepared in 10 ml of distilled water that was adjusted to an estimated concentration of 10^8 bacteria per ml using spectrophotometric assessment at 600 nm. Strict biocontainment precautions were taken during the experimental procedures. After each trial, the Biological Safety Cabinet Class BSC II (BSC II) (Holten Maxisafe 2000 Model 1-2, Denmark) was decontaminated using ultraviolet light for 3 hours followed by a 20 minutes exposure to aerosolized 70% (v/v) ethanol as previously described (Heidelberge *et al*, 1997) and 10 minutes exposure to 2% (v/v) Clorox. The BSCII was not used for at least 24 hours following an experiment to avoid any possibility of cross-contamination between experiments. To simulate aerosolization of different concentrations of *L. pneumophila*, the stock suspension of the organism was serially diluted from 10^0 to10^4 organisms/ml in 3 ml of sterile water and aerosolized using a nebulizer (Aerofamily, Italy) for 20 minutes in cabinet. The nebulizer was placed at the center of the workspace and mounted at a height of 60 cm from the work surface. Different concentrations of bacteria were used to determine the least amount of bacteria per liter of air that could be detected using the developed method. Baseline air samples were collected 20 minutes before the start of nebulization followed by differing concentrations of aerosolized *L. pneumophila* suspension. Air samples were collected for 20 minutes at a rate of 25 l/minute (total volume 500 l) following sample nebulization using an impinger air sampling device (Quickfit, England) connected to a suction pump (T100 M, rotary vane sampling pump, Cole-Parmer International, USA). Water from the impinger sampler was divided into two samples for *L. pneumophila* quantitation: 1) isolation on artificial media, and 2) quantitative real time PCR (qPCR).

Isolation and quantitation of *L. pneumophila* in impinger samples

The impinger water sample (7.5 ml) was centrifuged at 16,000*g* for 20 minutes at 4ºC, and the majority of the supernatant removed leaving approximately 0.5 ml remaining. The sample was mixed with 0.5 ml of 0.2 KCl-HCl buffer (pH 2.2), allowed to stand at 25ºC for 20 minutes, and 0.1 ml aliquot was inoculated onto WYOα medium containing cysteine (0.4 g/l), polymycin B (80 IU/ml), vancomycin (1 μg/ml), glycine (3mg/ml), and amphotericin B (80 mg/0.1 N KOH) and incubated for 7 to 16 days at 37ºC. The colonies were identified by sub-culturing on BCYE agar, with and without cysteine. These gram-negative bacilli, which grew only on BCYE agar with cysteine, but not on cysteine free medium, were presumptively identified as being *Legionella* spp organisms.

Quantitation of *L. pneumophila* using real time PCR

A qPCR technique for the quantitation of *Legionella* spp. DNA was developed using the Rotorgene 3000 platform (Corbett Research, Australia). Detection of *Legionella* spp DNA used a Taqman fluorogenic probe containing a 6-carboxyfluorescine (6-FAM) reporter and 6-carboxytetramethyl rhodamine (TAMRA) quencher. Primers and probe were purchased from Tib Molbiol GmbH (Germany) and designed using Primer3 software (http://www.-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi) based on Genbank deposited nucleotide sequence of 5S rRNA gene for *L. pneumophila* (Gnebnak accession X05081). The forward and reverse primer was 5′-TATAGCGATTTGGAA CCACCTGATAC-3′ and 5′-ATGAGGAAGCC TCACA CTATCATTG-3′ respectively, which generated a 84 bp PCR product. The probe had the following sequence, FAM-ATCTCGAACTCAG AAGTGAAACATTCC GC-TMR.

Genomic DNA was extracted from impinger water samples (7.5 ml) by concentrating the *L. pneumophila* by centrifugation at 8,000*g* for 20 minutes, and the pellet resuspended in 100 μl of the supernatant followed by centrifugation at 12,000*g* for 10 minutes. DNA was extracted from the resultant pellet using a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) following the manufacturers instructions and the isolated DNA

eluted in 100 μl of AE buffer (provided in the kit) was stored at 4ºC until analysis by qPCR. A "non-template control" that comprised sterile water without bacteria was included in each batch of samples. DNA samples were assessed for purity and concentration by spectrophotomety at 260 nm and 280 nm.

Real time PCR reaction was performed in a final volume of 20 μl that comprised 0.25 μM of each primer, 0.05 μM of probe, Master Mix (Biotools, Spain), containing 1 unit of Taq DNA polymerase, 0.2 mM of dNTP (dATP, dCTP, dGTP and dTTP/dUTP), buffer (Tris-Cl, pH 8.7 of KCl $(NH_4)_2 SO_4$, 2.5 mM $MgCl_2$ and sample DNA or non-template control. Each sample was analyzed in duplicate. Thermal cycling conditions were as follows: initial activation step were held for 3 minutes at 95ºC, followed by 40 cycles consisting of denaturation for 30 seconds at 94ºC and annealing/extension for 60 seconds at 60ºC. The limit of detection was determined using the cycle number threshold (Ct) at which normalized reporter dye emission is raised above background noise (corresponding to the mean baseline emission calculated for 3-15 cycles PCR). A Ct of >40 was considered negative.

A standard curve was constructed using diluted *L. pneumophila* genomic DNA stored in AE buffer. After spectrophotometer determination of the DNA concentration, the DNA was diluted to a concentration of 10^6 fg/μl and stored in small aliquots at -20ºC. qPCR sensitivity was determined by serial 10-fold dilution of the DNA standard (10^1-10^6 fg). The number of *Legionella* spp was calculate as follows:

No. of organisms/l = [DNA (pg) / N] x dilution factor / total air volume x genome molecular weight.

N = the number copy of 5s *rRNA* gene per genome = 3 (GenBank)

Dilution factor = 1:10 (*ie*, 5 μl from 50 μl of prepared DNA) and 1:2 (*ie*, half of water collected from impinger)

Total air volume = 500 liters

Legionella genome weight = 4.3 x 10^{-15}g (Bender *et al,* 1990).

Inter- and intra-assay variation was determined by performing the qPCR in duplicate. For comparison of sensitivity of the qPCR and culture methods. Chi-squared statistics was used to determine significant difference ($p<0.05$).

To prevent DNA contamination, each stage of the qPCR was physically separated into 3 areas: sample preparation area, pre-PCR area and post-PCR area. Three sets of pipettes were allocated specifically for each area and the aerosol resistant pipette tips were used. Also, the UV light was turned on for 30 minutes every time before starting the experiments. All reagents needed in PCR were aliquoted into several tubes, and these tubes were taken one at a time and discarded after use. In each experiment, positive and negative controls were also included.

RESULTS

Performance characteristics of qPCR

A standard curve (Fig 1) of diluted standard DNA was linear (r^2= 0.97) with a limit of detection of 10 fg (Ct = 39.95) (Table 1). Testing the diluted samples in duplicate gave a co-efficient of variation (CV) for intra- and inter-assay results of 0.34 and 3.90%, respectively. The qPCR was specific for *Legionella* spp as the eight non-*Legionella* spp failed to generate a Ct >40. The qPCR was significantly more sensitive than culture at *Legionella* concentrations of 10^1 to 10^4 organisms/ml (Table 2).

Impinger air sampling results

Using the air impinger sampler, *Legionella*

Table 1
Inter- and intra-assay variation of 5S *rRNA* gene detection for *Legionella* spp by qPCR.

	Co-efficient of variation (CV%)			
	Intra-assay (repeatability)		Inter-assay (reproducibility)	
DNA (fg)	Ct	CV	Ct	CV
10^6	27.00	0.05	28.20	4.34
10^5	30.36	0.06	31.88	4.88
10^4	33.66	0.08	34.80	3.33
10^3	36.39	0.60	38.42	5.42
10^2	39.51	0.73	39.06	1.14
10^1	39.95	11.11	39.93	0.05

Table 2
Comparison of detection rates of *Legionella* spp in spiked air samples.

Legionella (organism/ml)	% Positivity (*n* positive/*n* total)		Chi-squared[a] (p)	Median Ct (IQR)	Median DNA quantity (pg) (IQR)
	Culture	qPCR			
Air (no organisms)	0 (0/35)	0 (0/35)	>0.05	Not detected	Not detected
10^0	0 (0/35)	0 (0/35)	>0.05	Not detected	Not detected
10^1	23 (8/35)	57 (20/35)	0.003[b]	37.6 (35.6-38.8)	0.024 (0.006-0.043)
10^2	20 (1/5)	100 (5/5)	0.010[b]	39.5 (38.9-39.5)	0.262 (0.220-0.302)
10^3	20 (1/5)	100 (5/5)	0.010[b]	34.5 (33.7-25.5)	2.38 (2.14-4.01)
10^4	40 (2/5)	100 (5/5)	0.022[b]	26.3 (25.9-26.7)	31.1 (30.6-31.2)

[a]Difference between culture and qPCR
[b]Statistically significant difference in sensitivity

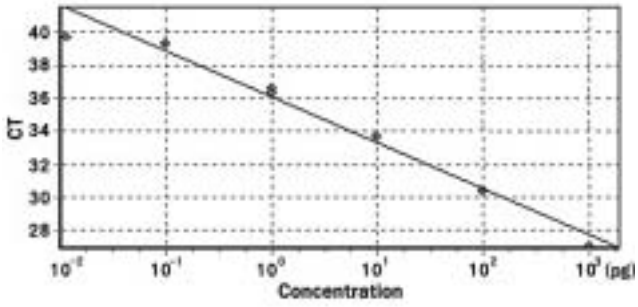

Fig 1–The standard curve of ten-fold serial dilution of *Legionell*a DNA of 1 ng to 1 fg (express as pg/reaction), generated by linear regression of the threshold cycle (Ct), using real time PCR (Corbett Research Australia).

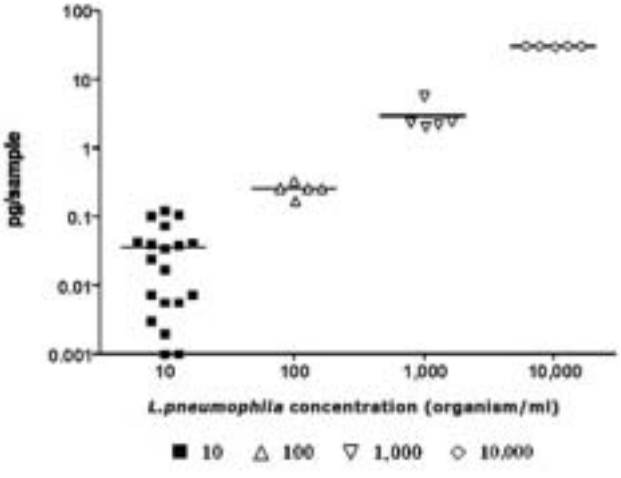

Fig 2–The correlation of spraying aerosol *L. pneumophila* concentration and the amount of DNA detected (pg/sample) from air samples using real time PCR (Corbett Research Australia).

organisms were reliably detected using qPCR (Table 2). *Legionella* was detected in 10^1 to 10^4 organism/ml of air samples as shown in Fig 2. At the lowest *Legionella* spp concentration (*ie* 10^1 organism/ml), DNA was amplified in 57% (20/35) of the samples by qPCR and the median DNA quantity was 0.024 pg (interquartile range (IQR) 0.006-0.043 pg) that ranged from 0.001 - 0.121 pg (equivalent of 1.5-185 organisms/500 l of air). Higher CV was noted at lower DNA concentrations (109%; 10^1 organism/ml) compared with higher concentrations (1.3%; 10^4 organism/ml).

DISCUSSION

The results presented here have demonstrated the utility of sampling using an impinger device, coupled with qPCR, for the detection of *Legionella* spp in air. The qPCR assay demonstrated increased sensitivity when compared with conventional culture methods for the testing of air samples collected by the impinger device.

The qPCR method presented here was, at least, as sensitive as previously reported assays using different gene targets. PCR assay targeting *mip* gene has reported detection limit of 10 fg of DNA (Wellinghausen *et al,* 2001), and that amplifying *Dot A* gene had a reported sensitivity of 29.9 fg (Yanez *et al,* 2005). Results from this study have demonstrated higher variation at lower concentration of samples and therefore results at the lower end of the concentration scale should be interpreted with caution.

The results presented here demonstrate that the impinger sampler is a highly efficient air sample collection method. The results of this study demonstrated a higher detection level when compared with results of previous studies from air samples that were collected from above a secondary aeration basin (<10^3 cfu/ml by PCR) (Palmer *et al,* 1995) and aerosol sampling around a *Legionella*-contaminated cooling tower (0.09 cfu/l of air by culture) (Ishimatsu *et al*, 2001).

The qPCR method presented here is specific and sensitive (can detect aerosol *Legionella* spp at 1.5-185 organisms/500 l of air). The qPCR is relatively rapid (4 hours) when compared to the traditional culture method, which requires a minimum of 7-10 days. However, the qPCR method is more expensive (US$ 12/sample). This approach could be used to study environmental airborne expulsion of infectious particles from *Legionella* spp contaminated sites and further field studies are required to validate the method.

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