

Full Length Research Paper

Comparative study on sampling methods for monitoring *Legionella* species in environmental water

Xiao-Yong Zhan, Chao-Hui Hu and Qing-Yi Zhu*

Guangzhou Kingmed Center for Clinical Laboratory, Guangzhou 510330, Guangdong, China.

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Infections caused by *Legionella* spp. are considered an emerging public health problem and are linked to high rates of mortality and morbidity, if not properly treated. This study analyzes 76 samples of water in Guangzhou with the aim of obtaining a more efficient means based on culture to isolate the genus *Legionella*. A comparative assessment of techniques recommended was performed on monitoring *Legionella* spp. in environmental water samples. Fourteen (14) sampling methods included in four categories based on concentration (filtration or centrifugation), acid buffer treatment (no acid treatment, treatment for 10 min), thermal treatment (no thermal treatment or treated for 30 min in 50°C) and heat enrich the samples in 37°C for 72 h were tested to identify their sensitivity. The four categories methodologies were assembled by different procedures and 14 methods were obtained to compare the efficiency for isolating *Legionella* in 76 water samples and in which *Legionella* were isolated from 42 water samples through these 14 methods. Merit and demerit of each single method was evaluated. Results suggest that a single category methodology for water sampling was insufficient to isolate *Legionella* spp. from water samples. Some *Legionella* positive water samples were missed through a single category methodology which made the results mistranslation. PSF category methods in this study were recommended methods and they could isolate *Legionella* species from 78.6% *Legionella* positive water samples; and if time was enough, PSF category methods combined with ERCT category methods to isolate *Legionella* from water samples are strongly recommended. *Legionella* were isolated from 41 water samples through these two category methods, it only missed 2.4% positive water sample. The results obtained, highlighted the importance of improving culture method for the identification of *Legionella* in water samples.

Key words: *Legionella*, environmental water samples, sampling methods.

INTRODUCTION

Legionella species, which are fastidious and ubiquitous worldwide in natural water environment such as rivers, pools, lakes and artificial water systems, are the causative

agent of legionnaire's disease (Wiin et al., 1998; Fliermans et al., 1981; Fraser et al., 1977; Declerck P et al., 2007). *Legionella pneumophila*, first recognized in

*Corresponding author. E-mail: zqy@kingmed.com.cn.

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Abbreviations: CT, Centrifugation for water concentration; ER, pre-treat samples in 37°C for 72 h for heat enrich; SF, sucking filtration for water concentration; PSF, sucking filtration and then add sterile water and scrape the membranes to collect bacteria; A, acid treatment; H, thermal treatment; AH, acid treatment and then thermal treatment; HA, thermal treatment and then acid treatment.

1977 following an epidemic of acute pneumonia in Philadelphia (Fraser et al., 1977), contributes to the majority of cases of legionellosis (Jarraud et al., 2007). In addition, at least 21 other species of *Legionella* have been related with human infections (Kümpers et al., 2008). *Legionella* bacteria do exist as free-living planktonic forms in the environment samples, intracellular parasites of protozoans, and/or inhabitants of mixed community biofilms (Taylor et al., 2009). The diversity types and the ubiquitous occurrence of *Legionella* in water environments or moist soil make it difficult to identify epidemic strains, and outbreaks of legionellosis have been associated mainly with contamination of artificial aquatic environments (Fields et al., 2002). The disease can be acquired by the inhalation of contaminated droplet aerosols or by microaspiration of contaminated water, makes it a legitimate public health concern as its fatality rate during an outbreak ranges from 5 to 30% in those who contract the disease (Declerck et al., 2007; Diederer et al., 2008).

However transmission of *Legionella* from person to person has never been observed, and the prevention needs to focus on the elimination of this pathogen from water and systems that produce aerosol (Borges et al., 2012). Thus, routine monitoring and testing of *Legionella* species in water system is of increasing importance for hazard prediction and the elimination of *Legionella* from potential infection sources (Mérault et al., 2011). The detection of *Legionella* in water samples has been usually performed using culture-based methods and is approved by the International Organization for Standardization (ISO 11731-2, 2004). However, it is complex and time consuming, need 3 to 10 days. Although there has been some rapid and convenient means such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) to detect *Legionella* in water samples, there powerless to distinguish live and dead bacteria cells limits their application (Delgado et al., 2009). Therefore, culture method still shows its vitality. The sampling and culturing methods including the sample collection and sample processing are the main factors influence the *Legionella* isolation (Ta et al., 1995; Vickers et al., 1987). To increase the isolation rate and efficiency to monitor *Legionella*, the ways to reduce the quantity of non-*Legionella* bacteria and to recover the *Legionella* bacteria in the samples are imperative. Thermal treatment which can be explained as incubating the collected water samples in 50°C or a higher temperature for several minutes is considered to be an effective means to reduce the quantity of non-*Legionella* as it can selective inhibit of non-*Legionella* bacteria (Mietzner et al., 1997; Kim et al., 2002). Based on acid-resistant characteristic of *Legionella*, acid buffer treatment is another significant means that can reduce non-*Legionella* bacteria in the water samples (Bopp et al., 1981; Vickers et al., 1987).

There has been some standard means for isolating

Legionella from water samples. These means include ISO 11731-2-2004 (ISO 11731-2, 2004), and "Procedures for the Recovery of *Legionella* from the Environment" (US CDC, 1995) approved by US CDC. The focal point of these standard means is on the sampling procedures. However, the basic procedure is common in each sampling methods and can be described as concentrate water samples first and then deal the concentrated water samples with acid buffer or heat. At last, the treated water samples were plated onto BCYE medium with selective supplements, and with several days (3 to 7 days) of incubation to obtain results.

To elevate the isolation rate and compare the merit and demerit of diverse methods for the water samples and finally find an optimal means recommend for the standardization, variables evaluated in this study including the concentration (filtration and centrifugation), acid buffer treatment (no acid treatment or treatment for 10 min), thermal treatment (no thermal treatment or treat for 30 min in 50°C) and heat enrich the samples in 37°C for 72 h. These variables were used independently or combined within the experiment to determine the advantages and disadvantages of each technique. The ultimate objective of present study was to recommend an optimal sampling method for the monitoring of *Legionella* in environmental water samples.

MATERIALS AND METHODS

Water specimens collect

A total of 76 selected 1000 ml water specimens were collected from different rivers, pools or lakes in Guangzhou with sterilized bottles. They were sampled for isolation of *Legionella* spp.. All water samples were obtained during November, 2008 to October, 2010. When samples were collected in sterilized bottles, treatment was immediately done within four hours in a microbiological safety cabinet to isolate *Legionella* from them according to strict sampling protocols described below.

Sampling protocols

The sampling protocols were based on concentration and different methods of sample treatment. The protocol based on ISO11731-2 was also used as a single method to be compared with the other methods originated in present study. These originated protocols have been grouped into four categories of sampling methods which were classified based on sample concentration method. The first two categories of methods were sucking filtration as water sample concentration, then gave the concentrated samples acid treatment, thermal treatment separately or combined (Figure 1). The third category of sampling processing was use centrifugation of the water specimens for 10 min in 3000 rpm/min as the method for sample concentration, then dealt with acid treatment or thermal treatment separately or combined (Figure 2). The fourth category of sampling method was first heat enrich samples at 37°C for 72 h as pre-treatment, then centrifuged the specimens for 10 min in 3000rpm/min as the method of sample concentration, dealt with acid treatment or thermal treatment separately or combined (Figure 3).

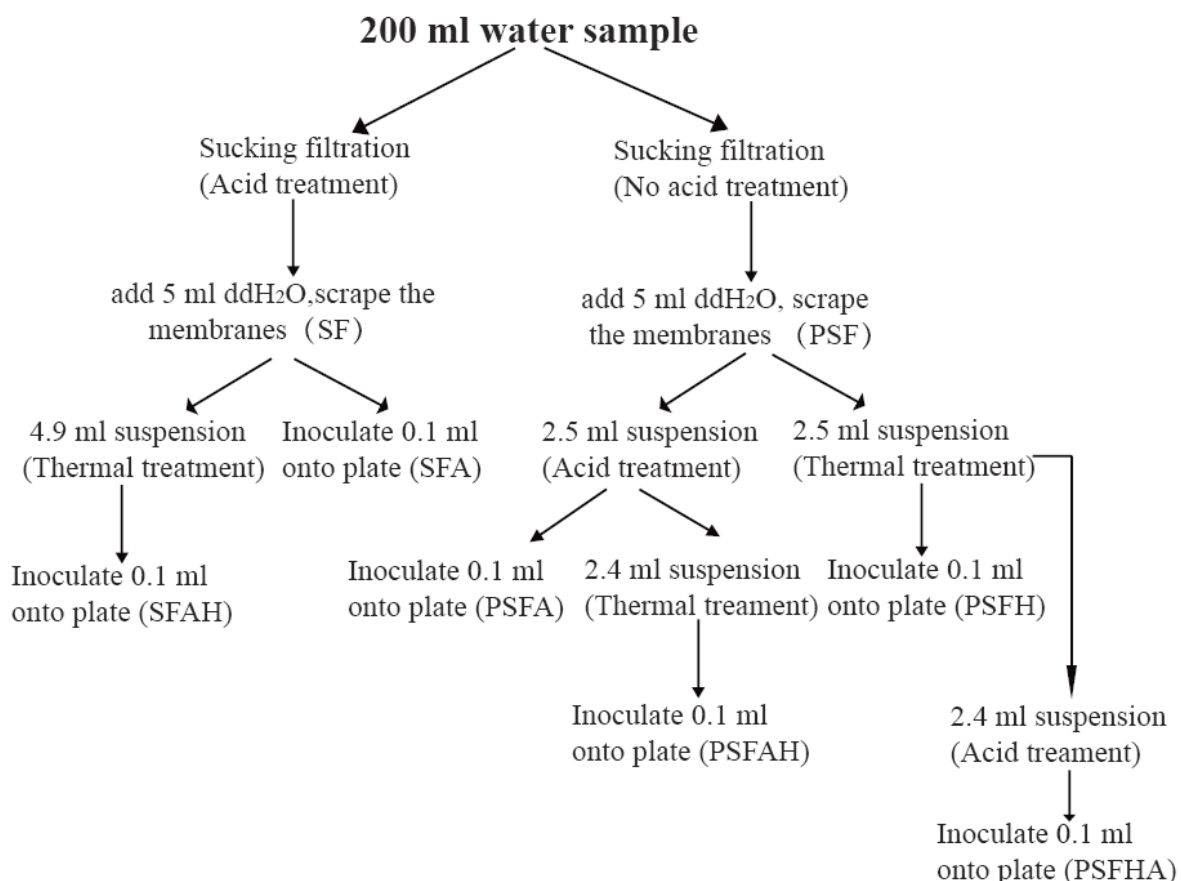


Figure 1. Overview procedures of the SF and PSF categories methods originated in present study.

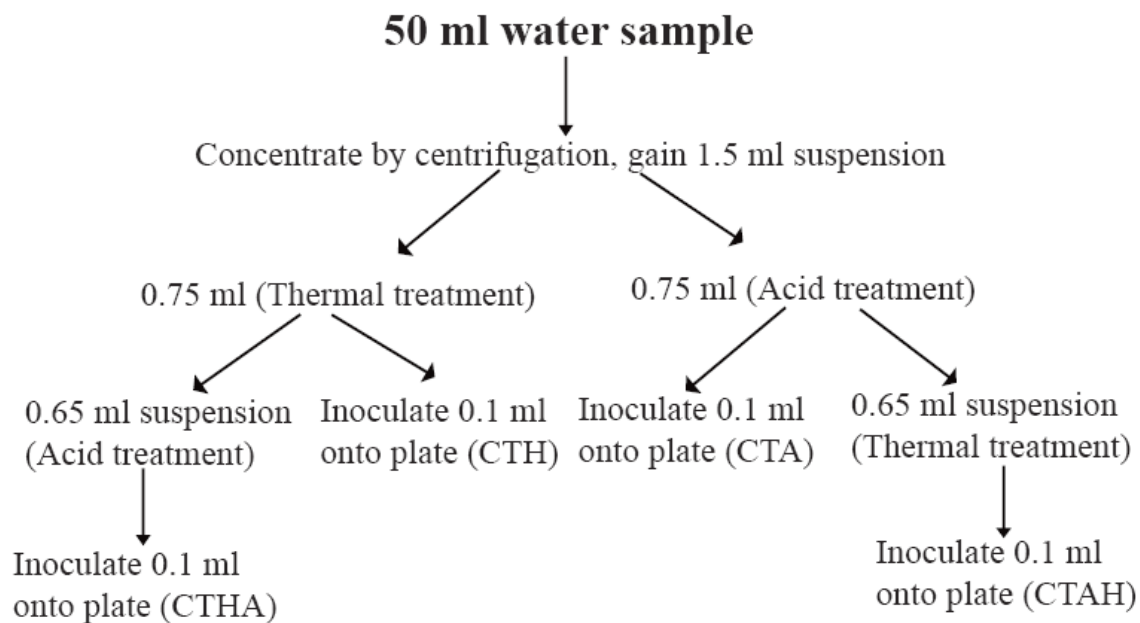


Figure 2. Overview procedures of CT category methods.

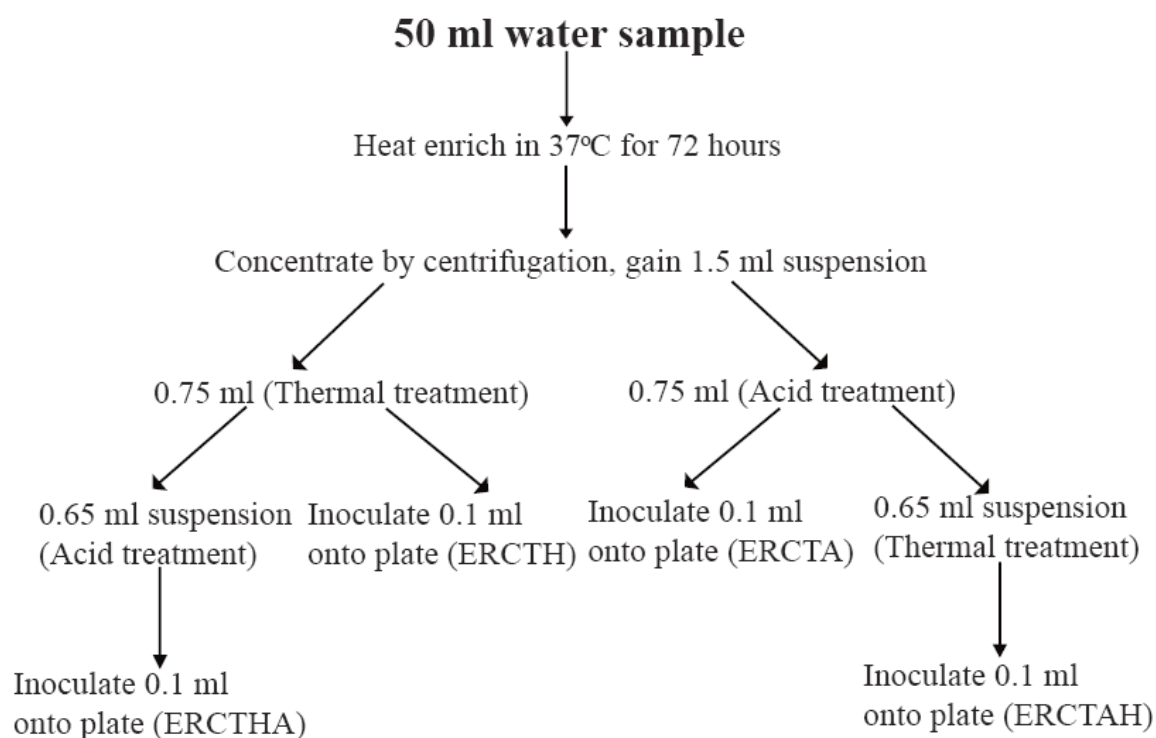


Figure 3. Overview procedures of ERCT category methods.

In the first two categories of originated protocol, the basic way for water sample concentration was to filter the water specimens with sucking filtration through 0.45 μm -pore diameter polycarbonate membranes by microfilm filtration system (Millipore Company, France). The differences between these two categories protocol were the further sampling process. One was immediately treat the filter membranes with acid buffer (0.2 M KCl-HCl, pH2.2) and could be described as follow: after filtration of the water sample, added 5 ml of acid buffer on top of the membrane in the filter, immediately filtered out the acid buffer, added another 10 ml acid buffer on the membrane and left it for 10 min, remove the acid buffer by filtration through the membrane and washed the membrane with 20 ml double distilled water (ddH₂O), added 5 ml sterile water or ddH₂O to the membrane, scraped filter membranes to collect bacterium and these methods were defined as SF category methods. The other category methods were described as follow: after water samples filtered, added 5 ml sterile water on the filter membranes, carefully scraped filter membranes without destroying it to collect bacterium from water specimens, the samples collected by the means mentioned were dealt with acid treatment or thermal treatment separately or combined and this category methods were defined as PSF category. Generally, the first two categories of the originated protocols could be illustrated as follows: 200 ml environmental water specimens were first concentrated by sucking filtration, acid buffer were immediately done by first adding 5 ml acid buffer on the top of the membrane and filtered it at once, then 10 ml acid buffer was added to the filter membranes and left it on the filter membranes for 10 min, then removed the acid buffer by filtration through the membrane, washed the membranes twice with 20 ml sterile water, added 5 ml sterile water to it, scraped the deposition on the

membranes, finally the 200 ml water samples were concentrated to 5 ml. 0.1 ml suspension from 5 ml concentrated samples was planted onto the BCYE agar with DGVP (SFA). The other 4.9 mL was continued to be treated by thermal treatment, after incubated in 50°C for 30 min, 0.1 ml suspension was taken and inoculated onto the BCYE agar with DGVP (SFAH). These two methods (SFA and SFAH) were based on ISO17731-2 and could be illustrated as Figure 1. The second category was somewhat the same as the first one. The only difference between these two categories was the specimens' further sampling processes. 200 ml water sample was also filtered, but acid treatment had not been done at once as in SF category methods. 5 ml sterile water was added on the top of filter membranes after sucking membrane filtration, deposition on the membrane was collected by a glass rod scrape. The collected 5 ml suspensions was divided into two parts, each was 2.5 ml and transferred to two sterile centrifuge tubes respectively, then centrifuged in 3000 rpm/min for 5 min to gain bacteria deposition and the deposition was re-suspension with 2.5 ml the acid buffer and left at room temperature for 10 min (PSFA) or re-suspension on 2.5 ml sterile water and incubated on 50°C for 30 min to do thermal treatment respectively (PSFH). If the samples from above PSFA continued treating with incubating on 50°C for 30 min, we called it PSFAH, so do the PSFHA, the procedures of sample treatment in this category were illustrated in Figure 1.

The third category methods could be illustrated as following: 50 ml environmental water specimens were centrifuged in 3000 rpm/min for 10 min, finally concentrated to 1.5 ml suspension. Then the suspension was dealt with thermal treatment or acid treatment, or first thermal treatment then acid treatment, or first acid treatment then thermal treatment. The acid treatment in this category could be

illustrated as follows: 1.5 ml suspension was divided into two equal parts with each 0.75 ml and transferred to 1.5 ml sterile tubes. Each part was centrifuged in 3000 rpm/min for 5 min, removed supernatant to gain deposition. The deposition was then suspended in 0.75 ml acid buffer, and left it at room temperature for 10 min. The acid buffer was removed by centrifugation; deposition was washed twice with 1.5 ml sterile water and finally suspended in 0.75 ml sterile water. 0.1 ml suspension was plated onto BCYE agar (CTA). The left suspension was done thermal treatment which was incubated in 50°C for 30 min and 0.1 ml of which was taken to plated onto BCYE agar with DGVP (CTAH). Water specimens also could be done thermal treatment first. 0.75 ml concentrated suspension was incubated in 50°C for 30 min for thermal treatment. 0.1 ml was plated after thermal treatment (CTH). The left suspension was centrifuged and the deposition was suspended with 0.75 ml acid buffer, left it at room temperature for 10 min and removed the acid buffer, washed with double distilled water twice, finally the deposition was suspended in 0.65 ml sterile water, plated 0.1 ml in BCYE agar with DGVP (CTHA). Details of these category methods were illustrated in Figure 2.

The last category of sampling method was somewhat like the third one. The difference between the two was the last category methods included a step of pre-treatment which was heat enrich in 37°C for 72 h (ER), and the rest sample process was the same as the third category. This category also included four methods which were ERCTA, ERCTAH, ERCTH, and ERCTHA. Procedure overview of each category method for the detection of *Legionella* in water was shown in Figures 1, 2 and 3.

Polymerase chain reaction (PCR) test for water samples

A nested PCR assay was used to detect *Legionella* in water samples. 50 ml water samples were centrifuged at room temperature in 3000 rpm/min for 10 min. Supernatant was removed; 100 to 200 µl DNA extracting solution was added to suspend precipitation. The suspension was transferred to a 1.5 mL tube and dealt with 70°C water bath heating for 15 min, then centrifuged at 10000 rpm/min for 10 min to collect supernatant. 5 µl of the supernatant which might include bacteria DNA was added to a total volume of 25 µl PCR system. The primers for first round PCR amplified a 386 bp portion from base 451 to base 837 of the 16S rRNA gene of *Legionella*. Forward primer 386F (5'-AGGGTTGATAGGTTAAGAGC-3') and reverse primer 386R (5'-CCAACAGCTAGTTGACATCG-3') were previously used elsewhere. JL Cloud et.al (Cloud et al., 2000) has described the specialty of this paired primers that it showed no cross-reactivity with any of these organisms, which included *Candida albicans*, *Streptococcus pneumoniae*, *Arcanobacterium haemolyticum*, *Staphylococcus aureus*, *Actinobacillus actinomycetemcomitans*, *Capnocytophaga granulosa*, *Eikenella corrodens*, *Pseudomonas aeruginosa*, *Moraxellacatarrhalis*, *Pasteurella multocida*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria sicca*, *Neisseria meningitidis*, *Bordetella bronchiseptica*, *Acinetobacter baumannii*, *Acinetobacter junii*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*. To enhance the sensitivity and specificity of detection for *Legionella* in water samples, a second round PCR has been carried out. 5 µL PCR product of the first round reaction was added to another PCR system with total volume of 25 µl. The primers of the second round PCR were primer 386F (5'-AGGGTTGATAGGTTAAGAGC-3') and primer 226R (5'-ATTCCACTACCTCTCCCATACTCGAGTCAACC-3') which amplify a 226 bp portion from base 451 to base 667 of the 16S rRNA gene of *Legionella*. These primers were originally designed and tested the specialty and sensitivity for *Legionella* (Zhan et al., 2010),

and showed no cross-reactivity with *Yersinia enterocolitica*; *Neisseria meningitidis*; *Salmonella typhi*; *Enterobacter cloacae*; *Klebsiellapneumoniae*; *Haemophilus influenzae*; *Proteus mirabilis*; *Enterococcus faecalis*; *Pseudomonas aeruginosa*; *Stenotrophomonas maltophilia*; *Escherichia coli*; *Staphylococcus aureus*. The thermal cycling profile of each round PCR assay consisted of an initial incubation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and finally 72°C for 5 min. After the second round PCR, 6 µl PCR product was used for agarose electrophoresis analysis.

Bacterial culture and medium

The DGVP supplement media including dyes, glycine, vancomycin, and polymyxin is effective in inhibiting background flora (Bopp et al., 1981). DGVP has the advantage of being the most cost-effective to use since it contains fewer antibiotics at lower concentrations than the other media and we used it in present study. All of the *Legionella* selective agar BCYE with DGVP and blood agar were prepared follow strict protocol and controlled for quality. Concentrated water samples were treated with acid or thermal described before and subsequently 0.1 ml of the suspension was spread on a 90 mm Petri dish containing BCYE agar supplemented with DGVP. The inoculated plates were then incubated at 37°C in an immaculate and humidified atmosphere with 2.5% CO₂ and observe every day, read at 3 to 10 days. Colonies were counted after 3, 5, and 10 days. Smooth colonies showing a yellowish or sometimes a yellow-green or grayish-white color were counted as suspicious legionellae to be confirmed. Suspected *Legionella* colonies were displaced and sub-cultured onto BCYE agar (without antibiotics) or blood agar for verification. The species identification of the primary verified *Legionella* was determined by molecular methods which were specific PCR for 16S rRNA gene and/or macrophage infectivity potentiator (*mip*) gene fragment and sequencing. Biochemical and serological tests were used for further confirming.

Statistical analysis

We followed a strict protocol for analysis, Chi-Square test was utilized in this study for comparing the category methods efficiency in isolation of *Legionella*. We considered P values of less than 0.05 significant. Graphpad Prism 6(GraphPad Software) was used for graphing. Statistical analyses were performed using SPSS 16.

RESULTS

Water samples

Legionella spp. was isolated from 42 water samples by the overall four category methods which was 55.3% of the total 76 water samples. The isolation rates of 7 different batches of water collected from different environmental water sites and obtained in different time range 22.2 to 100% (data not shown). The sampling protocols in this study were standardized for each water sample. However, different sampling methods had different isolation rates for *Legionella* spp. (Figure 4a and 4b; Tables 1, 2 and 3). It revealed that sampling method was an important factor that contributed to *Legionella* spp. isolation.

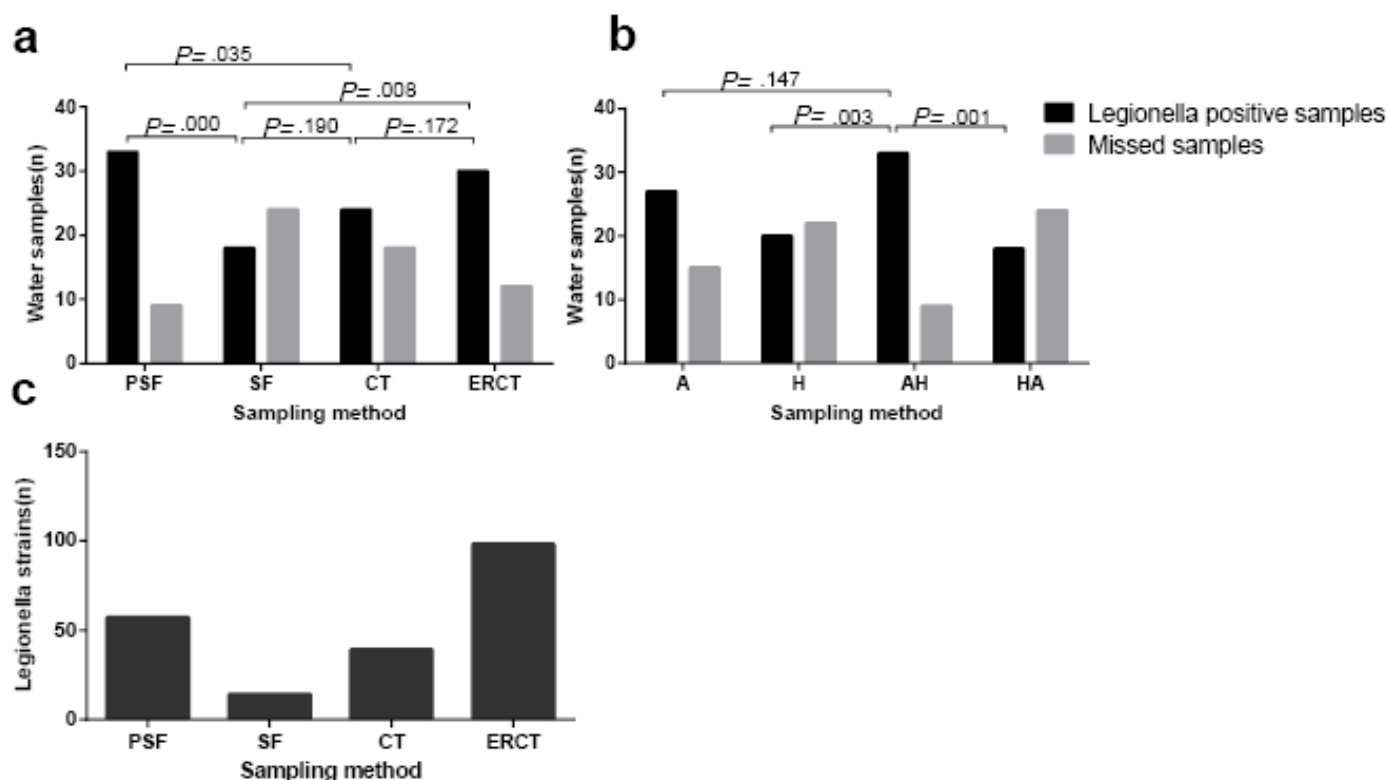


Figure 4. (a) Number of *Legionella* positive water samples processed by each category methods in the total 42 positive water samples. SF(n)=16, PSF(n)=33, CT(n)=24, ERCT(n)=30. The PSF category methods made more water samples positive compared to SF and CT categories methods ($P=0.000$ or 0.035 , Chi-square test). (b) Number of *Legionella* positive water samples processed by each method in the 42 positive water samples. A (n)=27, H (n)=20, AH (n)=33, HA (n)=18. AH method made significantly more water samples positive in the total 42 positive water samples than H and HA methods ($P=0.001$ or $.003$, Chi-square test). No difference had been shown between A and AH method. ($P=.147$) (c) Number of *Legionella* strains isolated from 24 water samples collected in Oct, 2010 in Guangzhou. SF (n)=14, PSF (n)=57, CT (n)=39, ERCT (n)=98. More *Legionella* strains were recovery through ERCT category methods than other three categories method. SF category methods is the most insufficient in recovery of *Legionella* strains.

Table 1. The number of *Legionella* positive and missed positive water samples through each method.

| Treatment method for water sample | Water concentration | | | | Acid/ thermal treatment | | | |
|--|---------------------|----|----|------|-------------------------|----|----|----|
| | PSF | SF | CT | ERCT | A | H | AH | HA |
| Number of <i>Legionella</i> positive samples | 33 | 16 | 24 | 30 | 27 | 20 | 33 | 18 |
| Number of missed samples | 9 | 18 | 12 | 12 | 15 | 22 | 9 | 24 |

Bacterial culture

The inoculated plates were observed every day. Abundant competitors were observed in plates after 24 h incubation in which the concentrated sample were treated only with thermal, indicating thermal treatment was not sufficient to inhibit competitors. A, AH and HA could sufficiently inhibit competitors, fewer than 15 bacterial colonies recovered in plates during 10 days incubation.

Almost all of the *Legionella* colonies appeared within five days incubation. About 90% *Legionella* strains were

recovered in plates in day 3, indicating BCYE agar with DGVP was efficient for *Legionella* growth.

Polymerase chain reaction (PCR) test for water samples

PCR is a simple method to detect *Legionella* in water samples. In this study, a nested PCR was utilized to define *Legionella* positive water samples. The two paired primers showed high specialty for *Legionella* which had

Table 2. The number of *Legionella* positive water samples gained based on water concentration categories.

| Category | Method | Number of positive water samples (n) | Number of positive water samples (n) |
|----------|--------|--------------------------------------|--------------------------------------|
| SF | SFA | 8 | 16 |
| | SFAH | 9 | |
| PSF | PSFA | 16 | 33 |
| | PSFAH | 23 | |
| | PSFH | 4 | |
| | PSFHA | 14 | |
| CT | CTA | 10 | 24 |
| | CTAH | 9 | |
| | CTH | 7 | |
| | CTHA | 12 | |
| ERCT | ERCTA | 18 | 30 |
| | ERCTAH | 18 | |
| | ERCTH | 12 | |
| | ERCTHA | 17 | |

Table 3. The number of *Legionella* positive water samples gained based on treatment categories(acid treatment and thermal treatment)

| Category | Method | Number of positive water samples(n) | Number of positive water samples (n) |
|----------|--------|-------------------------------------|--------------------------------------|
| A | SFA | 8 | 27 |
| | PSFA | 16 | |
| | CTA | 10 | |
| | ERCTA | 18 | |
| AH | SFAH | 9 | 33 |
| | PSFAH | 23 | |
| | CTAH | 9 | |
| | ERCTAH | 18 | |
| H | PSFH | 4 | 20 |
| | CTH | 7 | |
| | ERCTH | 12 | |
| HA | PSFHA | 14 | 18 |
| | CTHA | 12 | |
| | ERCTHA | 17 | |

been described by Cloud JL et al. (Cloud et al., 2000) and our research group (Zhan et al., 2010). From these seventy-six water samples, forty-two were *Legionella* positive according to the PCR results. However, three water samples in the total seventy-six water samples were

PCR negative but culture positive and another three were PCR positive but culture negative, and thirty-nine water samples were both PCR positive and culture positive. The study had shown good consistency between PCR and culture for detection of *Legionella* in water samples. This

Table 4. Detail of *Legionella* species isolated by each method from the 24 water samples in October, 2010 in Guangzhou.

| Category method | SF | | PSF | | | | CT | | | | ERCT | | | |
|--|----------------|------------------------|-----------------|-----------------|-------|------------------------|----------------|----------------|----------------|----------------|---|---------------------------------|----------------|------------------------|
| | SFA | SFAH | PSFA | PSFAH | PAFH | PSFHA | CTA | CTAH | CTH | CTHA | ERCTA | ERCTAH | ERCTH | ERCTHA |
| Number of <i>Legionella</i> positive water samples (n) | 6 | 6 | 11 | 9 | 2 | 8 | 8 | 6 | 6 | 5 | 13 | 15 | 9 | 14 |
| Number of <i>Legionella</i> strains (n) | 7 | 7 | 19 | 18 | 8 | 12 | 16 | 9 | 7 | 7 | 21 | 29 | 17 | 31 |
| <i>Legionella</i> species and number | LP: 6 LF: 1 | LP: 5 LF: 1 LG:1 | LP: 12 LF: 7 | LP: 14 LF: 4 | LP: 8 | LP: 5 LF: 6 LG:1 | LP: 9 LF: 7 | LP: 7 LF: 2 | LP: 6 LF: 1 | LP: 5 LF: 2 | LP: 15 LF: 1 LG:3 LM:1 LL:1 | LP: 18 LF: 1 LG:4 LL:6 | LP: 15 LG:2 | LP: 25 LG:3 LL:3 |

LP=*L. pneumophila*, LF=*L. feeleii*, LG=*L. gormanii*, LL=*L. longbeachae*, LM=*L. micdadei*.

result also reveals that some *Legionella* in water samples was non-culturable.

Species identification of the *Legionella*

Strict protocol was followed to identify *Legionella* species obtained by culture method. Specific PCR and sequencing for *16s rRNA* and/or *mip* gene was utilized to identify the species of *Legionella* strains isolated from 24 water samples obtained in October, 2010 in Guangzhou. Biochemical and serological tests were used for further confirming. Finally, 208 *Legionella* strains including the following 5 species: *L. pneumophila*, *L. feeleii*, *L. gormanii*, *L. longbeachae*, *L. micdadei* were obtained from 24 water samples. Detail was shown on Table 4.

Impact of water concentration to *Legionella* isolation

The water samples concentration methods were

summarized under four main categories methods, in which included fourteen specific methods. Finally, forty-two water specimens were *Legionella* positive by culture method and they were almost completely consistent with the nested PCR result, indicating that *Legionella* bacteria were really in these water samples. Of the forty-two *Legionella* culture positive water samples, *Legionella* could be isolated from thirty-three of which through PSF category methods including PSFA, PSFAH, PSFH and PSFHA methods, and thirty-one through PSFA plus PSFAH methods (Table. 1). In the PSF category methods, PSFH plus PSFHA methods which were done heat treatment first could only monitor 18 positive water samples and missed 24 positive samples.

Legionella could be isolated from 24 samples through CT category methods. Only 16 water sample could be detected *Legionella* through SF category methods. And this number was 30 through ERCT category methods. *Legionella* could be isolated from few water samples through SF category methods than other categories (the largest P value is 0.190, Chi-square tests, Figure

4a), indicating the significantly difference in the sensitivities of these categories methods in isolating *Legionella* spp. from water samples. More *Legionella* positive water samples could be detected through PSF category methods than through CT category method. ($P = 0.035$, Chi-square test, Figure 4a). Compared to PSF category methods, *Legionella* could isolate from other three water samples through CT category methods, but *Legionella* would not be detected in 10 positive water samples which could be detected through PSF category methods. *Legionella* could be detected in 30 samples through ERCT category methods and they would make other 8 water samples positive which were not positive by PSF category methods but would miss 3 positive water samples. SF category methods would make other 2 water samples positive but would miss 16 positive samples. In present study, a single sampling method such as PSFAH was also assayed and found it could only make few water samples positive, not sensitive to isolate *Legionella*, indicating one single sampling method was not sufficient to isolate *Legionella* from water

samples.

For instance, with PSFAH method, only 18 water samples were *Legionella* positive, 15 water samples missed, almost half of the total positive water samples. In order to enhance the sensitivity of the methods that utilized to isolate *Legionella*, two category methods were analyzed their efficiency to isolate *Legionella* from water samples. Results showed *Legionella* could be detected in almost all 42 *Legionella* positive water samples through PSF category methods combined with ERCT category methods. However, the isolation rate of SF category methods which represented the standard method originated from ISO17731-2 was extremely low, only 21.1% for the total 76 water samples. And for the 42 *Legionella* positive water samples, it would miss 26 samples. Detail of the result is shown on Tables 1 and 2.

Impact of water treatment to *Legionella* isolation

Water samples were first concentrated to enrich the bacteria by centrifugation, filtration, or heat enrichment which could be illustrated as category methods called CT, SF, PSF and ERCT in present study. To evaluate the impact of acid treatment or thermal treatment to *Legionella* isolation, 14 single methods were classified into another four categories methods which were A (acid treatment only, HCl-KCl buffer, PH 2.2 for 5 min or more), H (thermal treatment only, 50°C for 30 min), AH (acid treatment first and then thermal treatment) and HA (thermal treatment first and then acid treatment). Detail of each category methods efficiency was shown on Tables 1 and 3. It shows that *Legionella* could be isolated from 33 water samples through AH category methods, more efficient than HA and H category methods ($P = .001$ or $.003$, Chi-square test, Figure 4b). Many competitors could be observed after 24 h inoculation on plates on those samples treated with thermal only (H method), making it difficult to distinguish *Legionella* from competitors. While, fewer competitors could be observed during plate incubating on those samples treated with AH and H methods. This result indicated H category methods could not eliminate enough competitors but A and AH category methods could, led to relative higher isolation rates. An interesting fact was also observed in this study that HA category methods, which only with treatment sequence different to AH category methods but had an extremely lower efficiency in isolating *Legionella* ($P = .001$, Chi-square test, Figure 4b). Fewer bacterial colonies were found in plate by plating treated samples obtained from HA category methods, even the incubation time was extended to 12 days. It revealed that the processing sequence would influence the isolation. It is likely that the *Legionella* treated with thermal (50°C for 30 min) will become weakness to resist acid (Table 3).

Table 5. The number of *L. pneumophila* (L.p) and non-*L. pneumophila*(non-L.p) strains isolated through CT or ERCT methods from the 24 water samples in October, 2010 in Guangzhou.

| Method/ <i>Legionella</i> species | Number of L.p strains | Number of non-L.p strains |
|-----------------------------------|-----------------------|---------------------------|
| CT | 27 | 14 |
| ERCT | 73 | 25 |

Legionella strains isolated from water samples

To evaluate the impact of sampling methods to *Legionella* species isolation, number of *Legionella* strains of each species was calculated. Detail of which was shown in Table 4. *L. pneumophila* and *L. feeleii* could be isolated from these water samples through the overall four category methods (SF, PSF, CT, and ERCT). While, only by ERCT category methods, could *L. longbeachae*, *L. micdadei* be isolated from these water samples. The impact of water samples heat enrichment to *L. pneumophila* isolation was also compared between CT category methods and ERCT category method. It shown that water heat enrichment not only increased the efficiency of *Legionella* isolation (30 positive samples VS 24 positive samples, Figure 4a) but also increased the number of recovery strains(98 strains VS 39 strains, Figure 4c, Table 4). More *L. pneumophila* and Non- *L. pneumophila* strains were recovered from water samples during heat enrichment (Table 5).

DISCUSSION

Legionella species are pathogen of legionnaire's disease which appears as a mild respiratory illness to an acute life-threatening pneumonia or Pontiac fever (Edelstein et al., 1982; WHO, 2008). Epidemiological study demonstrates that legionnaire's disease is associated with environmental water system especially with the cooling tower system (Kusnetsov et al., 1994; Craun et al., 2010). Gold standard method for detection of *Legionella* species is based on cultivation (Den et al., 2004). This method allows the isolation and the quantification of cultivable *Legionella* species from environmental and clinic samples (Diederer et al., 2007). Isolating *Legionella* strains from environmental water samples is of great importance for epidemiological study (Den et al., 2004; Diederer et al., 2008).

Routine environmental cultures for *Legionella* in the water distribution system are recommended for risk assessments and transmission tracing that even if the hospital had not previously recognized cases of hospital-acquired Legionnaires' disease (Hoge et al., 1991).The cultivation procedure currently used to isolate *Legionella*

genus from environmental water samples is complex and time consuming and the effect sometimes is pending (Diederer et al., 2007). There has been many other means to detect *Legionella* in water samples such as PCR and Qpcr (Behets et al., 2007; Dusserre et al., 2008), with potential to detect infections caused by any *Legionella* species and serogroups, in addition to detect non-culturable *Legionella* and allow the manipulation of a large number of samples (Diederer et al., 2008; Jarraud et al., 2007). However, the major disadvantage of PCR or qPCR is the inability to evaluate the viability, in other words, it is not possible to distinguish between viable and nonviable microorganisms by the PCR technique, detecting only their presence or absence, while only viable bacteria are able to cause infections in human and represent an interest for public health (Delgado et al., 2009; Mérault et al., 2011).

In this study, different results between culture and PCR for detection of *Legionella* in water samples also revealed a fact that some *Legionella* in water samples was non-culturable. Culture method and PCR assay agreed well in present study, 39 water samples were both culture positive and PCR positive, indicating the sensitivity of culture method we utilized and highlighted the importance of improving culture method for the identification of *Legionella* in water samples. It also revealed PCR could be regarded as a useful complementary method for *Legionella* detection in water (Stojek NM et al., 2012).

As a result, although PCR and qPCR are the very fast and convenient means appeared, due to their natural disadvantages which are unable to distinguish live and dead *Legionella*, their application are limited (Morio et al., 2008). Therefore, improving the classic culture method is of greatly importance. The ISO11731-2 determines the standard method for detection and enumeration of *Legionella* in water (ISO 11731-2, 2004). The procedure of water sampling process is only the membrane filtration and acid treatment for several minutes with the water samples. It may be insufficient to inhibit the overgrowth of other accompanying bacteria, makes the culture and separation of *Legionella* difficult (Bartram et al., 2007). US CDC *Legionella* procedures referred the acid treatment, thermal treatment and heat enrichment, but didn't evaluate merit and demerit of each method (US CDC, 1995). The sampling and culturing methods including the sample collection and sample processing are the main factors which influence the isolation rate of *Legionella*. To improve the isolation rate, conditions of variable known to be favorable for the isolation of *Legionella* include concentration and different methods of sampling treatment were observed in present study.

Legionella species are generally present at very low or undetectable concentrations in freshwater and moist natural aquatic environments (Blanco et al., 2008). Thus, when working with environmental samples, it is usually necessary to use a concentration technique (centrifugation

and/or filtration) to enrich the microflora. This study demonstrates that compared to other three kinds of concentration (SF, CT, ERCT), the methods in PSF category which were first sucking filter water samples and then add appropriate amount sterilized water on the filter membrane of filter funnel, scrape filter membranes to collect bacterium from water sample was the best means for water concentration which would improve the isolation rate compared to other means (33 of 42, 78.6%). It would be explained as that PSF can collect more bacterium than other three kinds of concentration. ERCT method which was first incubated the water samples in 37°C for 72 h and then concentrated with certification had a higher isolation rate than that of CT method and it proved that heat enrich would improve the isolating, not only expressed as more *Legionella* positive water samples but also more *Legionella* positive strains.

The study on the 24 water samples collected in October, 2010 in Guangzhou indicated that this category method significantly improved the recovery of *Legionella* strains (Table 4). Almost half of the *Legionella* strains were isolated through ERCT category methods and only through this category method, could *L. longbeachae* and *L. micdadei* strains which were not recovery through other three categories methods were isolated, indicating the *Legionella* strains isolated through ERCT category methods were more diversified. We could put this fact down to the relationship between amoeba and *Legionella* (Bartie et al., 2003; Sanden et al., 1992), especially the relationship between amoeba and *L. pneumophila* (Christopher et al., 2010; Christopher et al., 2013). After 37°C incubation for 72 h, more competitors may be killed by nutrition exhausting but *Legionella* bacteria could use the nutrition from amoeba and love-lived (Brown et al., 1999; GoetzA et al., 1998).

The SF category methods represented ISO11731-2 method and it had the same procedures as ISO11731-2. However, this category methods had a lower sensitivity on detecting *Legionella* spp. from water samples and with which, only 16 positive water samples were detected, fewer than other categories methods (Figure 1a). SF category methods and PSF category methods had the same procedure in the water concentration. They are both sucking membrane filtration. The possible cause that led to different efficiency of *Legionella* isolating between SF and PSF category methods might interpreted as the SF category method could not do sufficient acid treatment. More overgrowth of other accompanying bacteria was observed in plates plating samples treated with SF category methods rather than with PSF category methods confirmed this hypothesis. Thus, PSF category methods are recommended rather than SF category methods.

The water samples should be treated with acid for 10 min to maximize the recovery of *Legionella* species and minimize the growth of competing microorganisms or treat

with 50°C for 30 min to eliminate the competitor (Bartram et al., 2007). The methods that used for water samples treatment can be used alone or jointly. Thermal treatment followed with acid treatment (AH) was of the greatest isolation rate, it could eliminate most of the competitor, and relatively pure bacterial colony of *Legionella* would appear in the plate within 48 h indicating this category methods was optimal treatment for water samples.

A single method had no high isolation rate for *Legionella* isolation. *Legionella* could only be detected in 23% *Legionella* positive water sample through PSFAH method, only 30.3% in the total 76 water samples and 54.8% in the total 42 *Legionella* positive water samples. This was the most effective single method in detecting *Legionella* in water samples (Tables 2 and 3); and the time needed for water samples processing in single method was not very shorter than category methods. Thus, a single method to isolate *Legionella* from water samples was not recommended. Although ERCT category methods did not significantly improve the isolation rate compared with CT category methods ($P=0.172$, Chi-square test, Figure 4a), when combined with PSF category method, these two category methods could make almost all *Legionella* positive water samples isolate *Legionella*. While, CT category method combined with PSF category method only makes 36 water samples *Legionella* positive. Therefore, ERCT category method plus PSF category method was superior to CT plus PSF, and combined with CT category methods, the missed 1 sample will be positive; that is, to say through these three category methods, all the 42 water samples will be positive.

Conclusion

This study demonstrates a comparing study of the sampling methods for isolation of *Legionella* from water samples. Fourteen (14) sampling methods based on four categories methods were evaluated in this study. Merit and demerit of each method was compared and demonstrated that one category methods was not sufficient to isolate *Legionella* species from water samples. PSF category methods were recommended to isolate water samples, and if time is enough, PSF category methods plus ERCT category methods to isolate *Legionella* from water samples are strongly recommended. Total time needed is about one week compared to four days of single PSF category methods. It only misses 2.4% of positive water samples.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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