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Detection of *Legionellae pneumophila* in Waters by Culture and Polymerase Chain Reaction

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Abstract

Legionella pneumophila (*L. pneumophila*) is the most common causative agents for all outbreaks of Legionnaires' disease. Prevention and control of Legionellosis requires surveying and monitoring of *Legionella* in the environment using conventional and modern technologies. The present study aims to compare detection of *L. pneumophila* in water samples using both culture and PCR techniques. A pre-enriched contaminated water sample was split into 13 subsamples. Culture and PCR tests were done from the subsamples after different intervals. The results showed a positive PCR result for *L. pneumophila* after 8 h of incubation. Also, *L. pneumophila* was detected by culture on non-selective BCYNE agar and selective GPVC agar after 5 and 6 days of incubation respectively. There was no significant difference between the non-selective BCYE- and the selective GVPC method. The PCR procedure was found more sensitive and differed significantly from the conventional selective GVPC method in isolation of *L. pneumophila* from water samples. It was concluded that pre-enrichment incubation allows the detection of *L. pneumophila* by PCR within a maximum of 12 h from the collection of water samples.

Keywords

Legionella pneumophila, PCR, BCYNE Agar, GPVC Agar, Water Samples

1. Introduction

Legionellae is a Gram-negative, aerobic, non-spore-forming, encapsulated bacillus. The genus *Legionella* comprises approximately 53 species. They are ubiquitous in natural fresh water environments such as lakes and streams. They pose a serious health risk into building water systems, and man-made aqueous environments which are rich with aerosolised water droplets [1]. *L. pneumophila* is

the causative agents for 90% of all outbreaks of Legionnaires' disease and Pontiac fever [2]. In addition, at least 20 other species of *Legionella* have been associated with human infections [3]. Transmission may be carried out from whirlpool spas, hot and cooling towers, air conditioning, and humidifiers. Prevention and control of Legionellosis requires surveying and monitoring of *Legionella* in the environment using conventional and modern technologies. Bacterial growth in the aqueous environment is sometimes exposed to stresses due to unfavorable conditions, so they adapt to such conditions by entering a temporarily non-cultivable state. In this state, they could only be grown into embryonated eggs or into amoeba (*such as Acanthamoeba castellanii*). Conventional methods include direct culture which is considered the "gold standard" and the classical method for detection of *Legionella* species using either selective or non-selective media such as, glycine-polymyxin B-vancomycin-cycloheximide (GPVC) agar or buffered-charcoal-yeast extract (BCYE) agar [4] [5]. However, the culture method is time-consuming because *L. pneumophila* is fastidious and too slow to grow, hence it is difficult to detect viable cells of *L. pneumophila* in routine culture methods, also the contaminants and impurities may interfere and hinder the *Legionella* growth [6] [7] [8] [9]. The rapid detection of *L. pneumophila* is essential to regulate the surveillance and control of *L. pneumophila* in supplied water. The application of PCR for the detection of *L. peumophila* DNA is useful for the early monitoring and controlling the organisms in water samples. This technique is cost-effective and reliable and has been described as useful tools for the detection of *L. pneumophila* in clinical and environmental samples. PCR has been considered a very promising tool for the detection of *L. pneumophila* DNA [10] [11]. Despite the advantages of conventional PCR, presence of inhibitors in water samples may produce false-negative results. In addition, conventional PCR is a qualitative, not quantitative method. To avoid such limitations, the chance of detecting *L. pneumophila* can be greater when using a pre-enrichment step prior to plating onto solid selective media or detection by PCR [12]. The present study aims to compare detection of *L. pneumophila* in pre-enrichment water samples using both culture and PCR techniques.

2. Materials and Methods

The study was carried out during period (January to June 2019) in the microbiology laboratory at the department of Environmental and Health Research, the Custodian of the Two Holy Mosques Institute for Hajj and Umrah, Umm Al-Qura University, Makkah, Saudi Arabia. Detection of the organism was undertaken after inoculation of water samples with *L. pneumophila* (ATCC 33152) by culture and PCR methods. Firstly, before inoculation, water sample was sterilized for 15 min at 121˚C to eliminate any bacteria potentially present in the water. Aliquots of 30 ml of an overnight culture on Buffered yeast extract broth (BYEB) medium of *L. pneumophila* isolates, containing 1×10^8 CFU ml^{-1}, was prepared. Next, the 30 ml of suspension was added to 1270 ml of the water sample to obtain a contaminated sample. Then, 130 ml of the sample was transferred

to a bottle, consisting of 1170 ml media (BYEB). The method was conducted in triplicates. The water sample was split into 13 subsamples (each subsample was of 100 ml). An uninoculated heat-treated water sample (100 ml) was used as a negative control. While incubation at 38˚C for 10 days, culture and PCR tests were done from each subsample, after 0, 2, 4, 8, 12, 24, 48 hours, and then after 3, 4, 5, 6, 7, and 10 days. From each water subsample, the 100 ml was concentrated by filtration using 47-mm-diameter polycarbonate membranes. In each sample, filter was placed in 10 ml of original water sample in a tube. Each tube was then vortexed for a few minutes, and the ten ml of solution was then concentrated to 1 ml by centrifugation at 10,000 g for 1 min, hence to get high number of organisms. A 0.1-ml volume of the concentrated sample was spread plated onto on non-selective BCYE agar and selective GPVC media, and incubated at 38˚C with 3% CO_2. All media were examined initially for the presence of *Legionella* bacteria. For PCR, firstly DNA was extracted by boiling method. Briefly, after concentration, 50 µL of water sample was placed into a tube, then subjected to boiling at 100˚C for five minutes. The sample was centrifuged at 3000 g for 10 minutes. The DNA-containing upper aqueous phase was recovered by centrifugation for 20 min, and genomic DNA was precipitated by ethanol [13]. The quantity and purity of extracted DNA were checked by spectrophotometry at 260 nm and the A260/A280 ratio. PCR was done by taking 50 µl of PCR mixture. The mixture contained 5 µl of DNA template, 1 µl (100 pmol) of each primer, a 25 µl of Taq PCR Master Mix polymerase (Qiagen, USA), and RNase free distilled water. The primers used in this study were obtained from IDT Integrated DNA technologies (IDT, Belgium). Mastercycler PCR machine (Eppendorf, Germany) was used for PCR reaction. The primers LEG 225 (5' AAGATTAGCCTGCGTCCGAT-3') and LEG 858 (5' GTCAACTTATCGCGTTTGCT-3') were used to amplify a 650 bp fragment of the 16SrRNA gene of the bacterium according to Hsu *et al.*, [14]. The cycling conditions were as follows: Denaturation (at 94˚C for 5 min), 35 cycles of denaturation (at 95˚C for 30 s), annealing (at 64˚C for 30 s), extension (at 74˚C for 20 s), and then 1 final extension cycle (at 72˚C for 5 min). The PCR products were viewed by gel electrophoresis (1.5%) and under UVP BioDoct It Imaging System after staining with ethidium bromide (2 mg/ml). Statistical analyses were carried out using the IBM SPSS version 23 software program (IBM® SPSS® Statistics, NY, USA). The culturing on the selective GPVC media was considered as "gold standard" and was assessed in McNemar's test.

3. Results and Discussion

The routine tests for the environmental monitoring of Legionella should be rapid and accurate and must be able to detect all living bacterial cells even those cannot be cultured. The present study aimed to compare detection of *L. pneumophila* in water samples using both culture and PCR techniques. The results showed that *L. pneumophila* was detected by PCR after 8 h of incubation (**Figure 1**). **Table 1** shows that 10 of the samples (76.9%) showed positive results by PCR test while **Table 2** shows sensitivity of 77%. Likewise, in many studies,

Legionella strains including *L. pneumophila* was isolated in the water samples [15] [16] [17].

Table 1 also shows *L. pneumophila* was detected by culture on non-selective BCYNE agar after 5 days of incubation. The total number of the positive samples by culturing on non-selective BCYNE agar was 4 (30.7%) with sensitivity of (30.7) as shown in Table 1. Also *L. pneumophila* was detected by culture on selective GPVC agar after 6 days of incubation. The control sample (uninoculated heat-treated water) was found to be negative. Edagawa et al., reported that 20.0% water samples from buildings were positive by culture, qualitative PCR or both methods [18]. With respect to the positive Legionella cultures, there was no significant difference between the non-selective BCYE- and the selective GVPC media (p-value = 0.137), however, the PCR method differs significantly from the conventional selective GVPC media (p-value = 0.008) (Table 2). Table 2 also shows that, the use of non-selective BCYE- method in the isolation of *L. pneumophila* from water samples was less sensitive (30.7%) than PCR procedure (77%), and that was consistent with previous observations [19]. There was even, a study in which the culture method showed negative growth in all samples [20]. The lowest colony counts detected by the non-selective BCYE- and the selective GVPC media were 5.2×10^2 cfu/ml (after 5 days' incubation at 37 C) and 6.5×10^2 cfu/ml (after 6 days' incubation at 37 C) respectively. Many factors may explain the low sensitivity rate of detection *L. pneumophila* by culture in water samples, such as: the presence of viable but non-culturable cells, loss of viability of bacteria after collection, and low concentration of legionellae in the samples [21]. Similar observations were reported by other authors [22] [23] [24]. The reason for the significant discrepancy between PCR and culture results for *Legionella* in water samples is the greater positivity rates for PCR than culture [25].

Table 1. Detection of *Legionella* spp. in water subsamples by culture and PCR techniques.

Sub sample	Time	Non-selective BCYNE agar cfm/ml	Selective GPVC agar cfm/ml	PCR
1	0 hours	0	0	−ve
2	2 hours	0	0	−ve
3	4 hours	0	0	−ve
4	8 hours	0	0	+ve (faint)
5	12 hours	0	0	+ve
6	24 hours	0	0	+ve
7	48 hours	0	0	+ve
8	72 hours	0	0	+ve
9	4 days	0	0	+ve
10	5 days	5.2×10^2	0	+ve
11	6 days	7.5×10^2	6.5×10^2	+ve
12	7 days	2.1×10^3	3.4×10^3	+ve
13	10 days	1.5×10^4	2.2×10^4	+ve
Control	Everyday	0	0	−ve

Table 2. Comparison of pre-enrichment methods with BYEB medium for isolation of *L. pneumophila* from water samples.

		PCR		Non-selective BCYNE agar		Total
		+	-	+	-	
Selective GPVC agar	+	3	0	3	0	3
	-	7	3	1	9	10
Total		10	3	4	9	13
P-value		0.008		0.137		
Sensitivity		77%		30.7%		

Figure 1. Lane 1; Negative control. Lane 2: negative LEG gene of *L. pneumophila*. Lanes 3, 4, 5, and 6: positive LEG gene of *L. pneumophila* (650 bp). Lane 7: Positive control positive. Lane M: 100-bp DNA ladder.

4. Conclusion

There was no significant difference between the non-selective BCYE- and the selective GVPC media, however, the PCR method differs significantly from the conventional selective GVPC media. The PCR method after pre-enrichment is rapid and simple for the detection of *L. pneumophila* in water samples.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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