British Microbiology Research Journal
4(12): 1531-1540, 2014
ISSN: 2231-0886

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Detection of Pathogenic *Legionella pneumophila* in Tap Water and Water of Newborn Incubators in Hospitals of Guilan Province by PCR of *mip* Gene

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MAJM and HH designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the analyses of the study. Author SAM performed the sampling, author BST and MN managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2014/9830
Editor(s):
(1) Alok K Upadhyay, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA.
(2) En Tao Wang, Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, State Key Laboratory of Agrobiotechnology and College of Biological Sciences, China Agricultural University, Beijing, China.
Reviewers:
(1) Anonymous, Umm Al-Qura University, Saudi Arabia.
(2) Graciela Castro Escarpulli, Laboratorio de Bacteriología Médica Escuela Nacional de Ciencias Biológicas Instituto Politécnico Nacional Plan de Ayala y carpio s/n Colonia Plutarco Elías Calle Del Miguel Hidalgo, México.
(3) Anonymous, Maria Curie-Sklodowska University, Poland.
Peer review History: http://www.sciencedomain.org/review-history.php?iid=605&id=8&aid=5866

Original Research Article

Received 3rd March 2014
Accepted 13th June 2014
Published 22nd August 2014

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ABSTRACT

Aims: This study is designed to determine the frequency of *Legionella pneumophila* in cold and warm water as well as water containers of newborn incubators in Guilan province hospitals, Iran, using amplification of the macrophage infectivity protein gene (*mip* gene) by PCR.
Study Design: Cross sectional study.
Place and Duration of Study: The present study was performed in the Cellular and Molecular Research Center, Guilan University of Medical Sciences between June 2011 and July 2012
Methodology: Samples were collected directly in sterile containers, concentrated in centrifuge, transferred to yeast extract broth containing L- cysteine, Fe^{2+}, Glycin and vancomycin and incubated for 3-4 days. DNA was extracted by using the boiling method and PCR was performed to search Legionella and *mip* gene using two pairs of primers. Contamination with other bacteria was evaluated in all negative samples using universal primers of 16S rRNA gene.
Results: About 8.5% of the samples had *L. pneumophila* including 11% of the incubators and 5.8% of both hot and cold tap water. The *mip* gene was found in 2.8% of the samples. One third of the incubator and one half of the hot water habited *L. pneumophila* had the *mip* gene but it was not found in cold tap water samples. About 87.2% of the negative samples showed bacterial contamination as revealed by PCR with primers of 16S rRNA gene.
Conclusions: This study indicates that in spite of using distilled water for incubators, *L. pneumophila* contamination is considerable and other bacterial contamination is very high. It may be related to the length of time that water remains in an incubator container which is a predisposing factor for both biofilm formation and the growth of water microflora. It seems that the high temperature of hot water system and the high rate of free residual chlorine in tap water system are the main causes of low rate of Legionella contamination but are ineffective on contamination with other bacteria.

Keywords: Legionella pneumophila; mip gene; PCR; hospital tap water.

1. INTRODUCTION

Legionella is a part of microflora in some aquatic environments, which contained 60 serovars [1] with some pathogens for human. They are usually transferred to host by inhalation of contaminated aerosols and cause sporadic and epidemic pneumonia [2]. About 85.0% of these infections are caused by *Legionella pneumophila* [3,4].

Hospital outbreaks are usually associated with contamination of water sources [5]. Water contamination of respirators and incubators of newborns are the main sources of transfer [6,7]. The cooling tower and the air condition system have also been reported to be sources of several outbreaks [8]. Manmade water systems especially hot water systems are main sources of Legionella [9]. These bacteria survive in biofilm and resist against chlorine and other disinfectants [9].

Culture is the most usual method for the surveillance of environmental waters and it is suitable for isolation and microbial counting [8,10] but it is slow and time consuming especially for clinical diagnosis [11]. Waters supplies containing 104-105cfu/ml L

pneumophila are risky for human [8]. Epidemiological data indicate that most of the outbreaks occur with this bacterial concentration in waters [12]. In addition, Legionella can survive in (facing with) poor nutrition, osmotic stress and oxidative stress (chlorine) with changing the viability status to the viable but non cultivable (VBNC) organisms which are able to grow again in suitable condition [13,14].

In recent years more sensitive and rapid assays are being used. PCR is an attractive substitute for culture and for Legionella diagnosis in clinical specimens and water samples [15] which are applicable for VBNC diagnosis as well [14]. The mip gene coding a Surface-Exposed Peptidylproline cis-trans-Isomerase is the main pathogenic gene of *L. pneumophila* [16] so the direct diagnosis of pathogenic Legionella is possible by using a PCR assay for detection of mip[17].This assay is the best way to overcome psedonegaive problem facing with culture whose sensitivity is 50-60% [18].The Aim of the present study was to determine the frequency of *L. pneumophila* in cold and warm water as well as water containers of newborn incubators in hospitals of Guilan province, Iran using the mip gene amplification by PCR.

2. MATERIALS AND METHODS

2.1 Water Sampling

During April- June 2012, 140 water samples (about 50ml for each) were aseptically collected in 60 ml plastic containers from 33 hospitals, from incubators of newborn wards and from hot and cold outlets after the water had run for 5min. Potable water is supplied to the hospitals by the municipal system. The water has detectable free residual chlorine upon arrival at the hospital, where it receives no additional treatment. Cold water is pumped to each building where it is heated. All water samples were concentrated 20-fold (by dividing with 10ml in 5 tubes, centrifugation with12000 rpm for 30min in 4ºC, discarding supernatant, resolving sediment in 0.5ml of the solution). The concentrated water samples (2.5ml) were collected in a sterile 15ml falcons and treated in 50ºC for 30 min to reduce other contaminants.

2.2 Enrichment of Bacteria

Each of the treated water samples was inoculated in 5 ml liquid medium containing yeast extract 10g/l, L-cystein 0.04%, ferric pyrophosphate 0.25g/l, glycin 0.3% and vancomycin 5µg/ml with final pH 6.9 and incubated in 35ºC for 3-4 days for bacterial growth. The selectivity of the medium was subsequently improved by the incorporation of vancomycin and glycin. This selective medium should facilitate the recovery of Legionellaceae from environmental sources [17]. Then cultured samples were divided over 1ml in volume and centrifuged in a Sigma model 3k30 centrifuge at 12,000 rpm in 4ºC for 20min. The supernatant was discarded and the pellet was suspended with 1 mL of sterile water before DNA extraction.

2.3 DNA Extraction and Primers

DNA extraction was performed by using boiling method (96ºC for 12 minutes) as following procedure: 1)One ml of the cell suspension in water used for lysis, 2) after the heating, the lysate was transferred to temperature -20ºC followed by adding the cold isopropanol to make precipitation with cold isopropanol. The extracted DNA was either used immediately for PCR or stored at -20ºC until analysis. The positive control consisted of genomic DNA for L.

pneumophila strain NCTC 11192. The negative control was pure water used for PCR mix. Three pairs of primers were used in this experiment: L. pneumophila - species specific primers (Lp-16S), F: 5- CCTGGGCTTAACCTGGGAC and R: 5´-TTAGAGTCCCCACCATCACAT--3´ Previously used by Pourcel et al. [19] for amplifying a specific portion of the 16S rRNA gene, *Legionella pneumophila*- mip gene specific primers (Lmip R) F: 5- ATGATAGCTTATGACTGGTA -3 R: 5- TTCCTTTGTTCACTCAGTAT -3 previously used by Engleberg et al. [16] and Higa et al. [20] for amplifying middle portion of mip gene, and finally the universal primers for bacterial 16SrRNAF-5/-GGATTAGATACCCTGGTAGTCC-3/ and R-5/-TCGTTGCGGGACTTAACCCAAC-3/ previously used by Kariyama [21] for amplifying a conserved portion of 16S rRNA gene.

2.4 PCR Conditions

Five micro liters of the extracted template DNA were used in a 20µl reaction mixture that included 10µl of PCR premix [Prime Taq Premix (2x), Chorea Lot No 201208], 0.5µl each primer, and 4µl of ddH2O. Cycling conditions for amplification of *L. pneumophila* - species specific fragment began with an initial denaturation at 94ºC for 5min and then 40cycles consisting of 94ºC for 40s, 44ºC for 40s and 72ºC for 40s were followed by a final extension at 72ºC for 7 min. Cycling program for *L. pneumophila mip* gene amplification was the same but the annealing temperature was 46ºC. Annealing temperature for 16S rRNA amplification with the universal primers was 47ºC with the same cycling program. Electrophoresis of amplified product was performed on agarose gel (1.5% w/v) by 125 volt for 45 minutes and analyzed by GelDoc Transluminator system (Vilber Lourmat model).

3. RESULTS AND DISCUSSION

One hundred and forty samples were collected from 33 hospitals including 72 samples from incubators, 34 samples from cold tap water outlets and 34 samples from hot water outlets. The incubation time for hot and cold tap water samples was 4 days and for incubator samples was 3 days. Totally 12 samples were positive for *L. pneumophila* (8.5%) in which 4 samples (33.3%) had *mip* gene (2.8% of all samples) (Table 1, Figs. 1 and 2).

Table 1. Sampling information and *Ligionella* detection results by PCR

Hospital	Sample		Incubation	Amplification result		
	type	code	time (d)	*Legionella*	*mip* gene	Bacteria
Shahidansari Rudsar	I	SR1	3	+	+	+
	I	SR2	3	+	-	+
	I	SR3	3	+	-	+
	I	SR4	3	+	-	+
Aria Rasht	T	AR1	4	+	-	+
	H	AR2	4	+	-	+
Shahidbeheshti Astara	H	SA3	3	+	+	+
Razi Rasht	T	RR2	4	+	-	+
Alzahra Rasht	I	AR4	3	+	-	-
Imamkhomeini Anzali	I	IA2	3	+	+	+
Salamat Rostamabad	I	SR1	3	+	-	+
Shahid Amini	I	SA3	3	+	+	+
Saeyedalshohada Lahijan	H	SL1	4	+	-	-

**. T: tap water; H: hot water; I: water of incubator*
+, positive; -, negative

Positive cases for *L. pneumophila* in incubator samples were 8 of 72 (11.1%) in which 3 samples (37.5%) had mip (4.2% of all incubator samples). For cold tap water, 2 of 34 samples had *L. pneumophila* (5.8%) that had no *mip gene*. Contamination rate of *L. pneumophila* for hot water samples were the same (5.8%) but one half of bacteria had *mip* (2.9% of all hot water samples). About 85.1% of all the negative samples (109 of 128) were positive in PCR with universal primer 16S rRNA indicating the presence of other bacteria (Fig. 3). This contamination rate was 87.1% for incubator samples, 82.7% for hot water and 93.5% for cold water samples. It seems that they are gram negative bacteria because there was 5µg/ml vancomycin were used in the enrichment medium that could inhibited most gram positive bacteria.

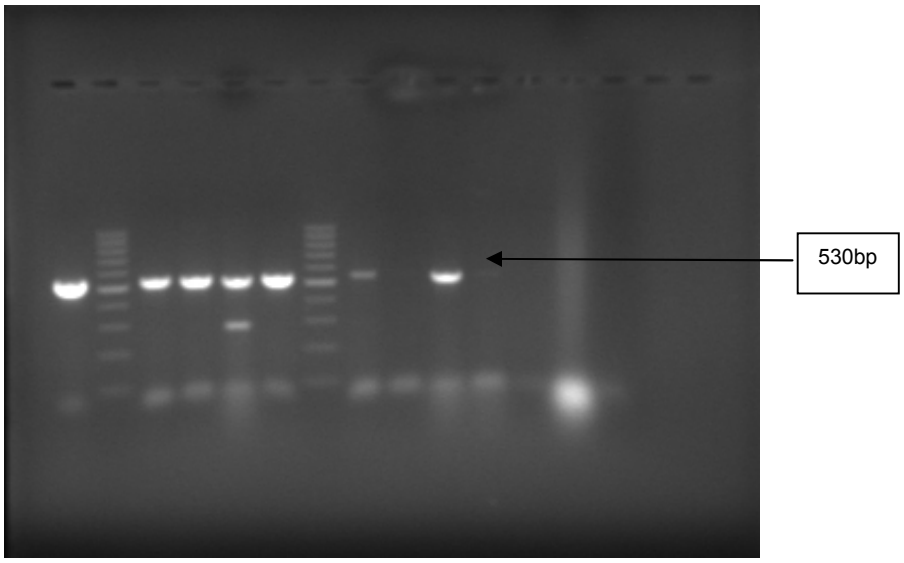
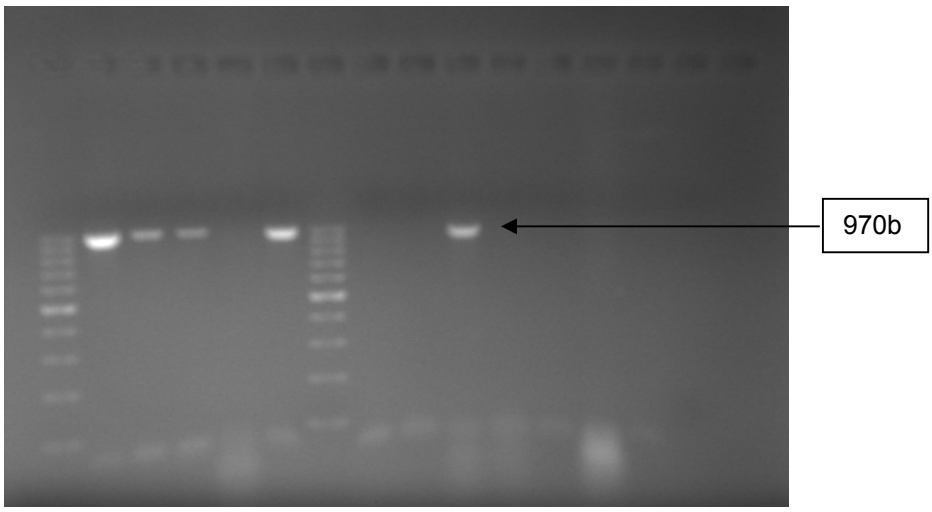


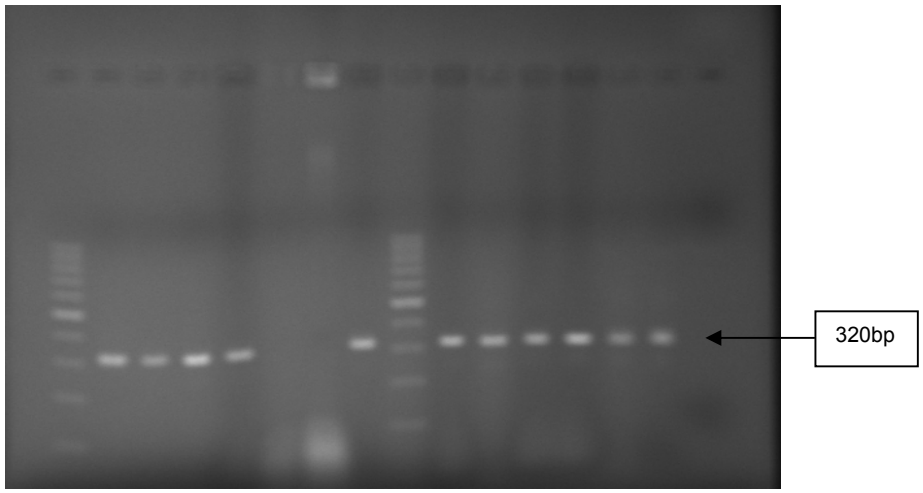
Fig. 1. Results of PCR with *L. pneumophila* - species specific primers (Lp-16S), for 13 samples. Line 1: positive control (*L. pneumophila* type strain NCTC 11192) with 530bp specific band. lines 2 and 7: Ladder (100 to 100 bp), line 3, 4, 5, 6, 8, 10, and 11 :positive samples with 530bp specific bands. lines 9, 12, 13, 14, 15: negative samples. line 16: negative control (water)

In the present study, we used liquid medium to enrich the bacterial number. In the enrichment procedure, glycin was added and heat treatment was employed for inhibiting growth of other bacteria. Medium with 0.3% glycin was tried by Wadosky [22] and is confirmed for isolation of Legionella from environmental samples. Legionella can tolerate and even replicate in 50ºC so its viability remains unchanged in hot water buildings' system [23] but temperature higher than 50ºC is lethal for Legionella and temperatures over 55ºC can be used to control Legionellae in water supplies [23]. We found that a low rate of *L. pneumophila* contamination in hot water samples might be associated with high temperature of hot water tanks (60ºC in most studied hospitals). The study of Legani et al. Presented that there is adverse association between temperature degree and Legionella count in hot water [24]. Darelid et al. reported that after adjusting temperature of hot water tank higher than 55ºC, incidence of hospital pneumonia decreased dramatically [25].



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 2. Results of PCR with *L. pneumophila* - mip gene - specific primers (Lmip R), for 11 samples. Lines 1 and 7: 100bp Ladder(from 100 to 1000),Lines 2: positive control (*L. pneumophila* type strain NCTC 11192) with specific band, lines 3, 4, 6, and 10: positive samples with 970 bp specific bands, lines 5, 8, 9, 11, 12, and 13: negative samples, line 14: negative control (water)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 3. Results of PCR with Universal Primer for bacterial 16S rRNA gene for 13 samples. Line 2: positive control (*L. pneumophila* type strain NCTC 11192) with 320bp specific band; lines 1 and 9: 100bp Ladder (from 100 to 1000bp); lines 3, 4, 5,,8, 10, 11, 12, 13, 14 and 15: positive samples with 320bp specific bands; lines 6, 7 and 13: negative samples, line 16: negative control (water)

Many hospitals prefer adjusting the water temperature ≤50ºC to avoid blowing with hot water. So most cases of *L. pneumophila* contamination in hospitals are associated with hot water aerosols. Meenhorst et al. studied on 21 *L. pneumophila* isolated in a university hospital in Netherland and reported that the serogroups of respiratory specimens isolates and water isolates were the same [26]. In the study of Reinthaler et al. on 210 hot and cold water samples from 21 hospitals, 34% of the samples were positive for *L. pneumophila* [27] that is more than of our result (8.5%). Study of Borella et al. on 119 hot water samples of hotels in Italy showed high rate contamination with *L. pneumophila* (45.8%) [7]. This study showed that there is adverse association between bacterial count of water samples and free residual chlorine. It seems that the low rate of Legionella contamination in cold tap water in our study is related to the high rate of free residual chlorine due to hyper chlorination of municipally water system that is usual here. The study of Tison et al. showed that Legionella count of water decreases up to 1000 fold by chlorination [28]. The study of Lin et al. [29] showed that increasing temperature, hypercholorination and using ultra violet are effective procedures to disinfect hospitals water systems. Palmer et al. also reported that viable count of Legionella decreased by chlorination [30]. The study of Joly et al. [31] also showed that temperatures over 60ºC and free residual chlorine decreased Legionella such that it would not be detected by Quantitative Real –Time PCR.

Another important point is volume of water sample. The more volume of water, the more bacterial count is expected. Studies of July [31] and Borella[7] showed that Legionella cut off count in water to be diagnosed by PCR is ≤103Cfu/L. We used 50ml sample for the test so the cut off for this volume must be ≤50 CFUs. We tried to capture total bacteria by using high speed centrifugation (12000rpm in 4ºC) and then we used culturing in an enriched medium to increase the bacterial count before performing PCR. Several studies showed that PCR is more sensitive than culture [32-34], so the low volume of samples was not a problem in our study.

The study of Yaslianfard et al. [35] on 52 water samples of 7 hospitals in Tehran, Iran, showed 9.6% legionella contamination that is greater than our result on tap water (5.8%)because half of their samples were taken from showerhead which usually has biofilm that is shelter for Legionella.

Another significant finding in our study is the high rate contamination with other bacteria in most samples which were negative for Legionella (85.1%) that is compatible with Stojek´s studies (2008 & 2011) which showed 79.1% and 87.2% contamination of tap water samples with gram negative non *Entrobacteriacea* mostly *Pseudomonas aeruginosa* [36,37].

4. CONCLUSIONS

We found that, *Legionella pneumophila* contamination is considerable and other bacterial contamination is very high in incubators water. It may be related to the length of time that water remains in an incubator container which is a predisposing factor for both biofilm formation and growth of water flora. It seems that the high temperature of hot water system and the high rate of free residual chlorine in tap water system are the main causes of the low rate Legionella contamination but are ineffective on contamination with other bacteria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=605&id=8&aid=5866