Molecular Detection of *Legionella pneumophila* within Culture-Negative Samples

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Abstract

Introduction: *L. pneumophila* is the most important cause of legionaires, disease, which is currently reported either as a nosocomial or community acquired infection. Rapid and reliable diagnosis of legionella has hampered the epidemiological studies and disease control activities. In spite of good sensitivity and specificity, the culture method encountered serious shortcomings. Polymerase chain reaction (PCR) has been used to detect the viable but non-culturable legionella. In the present investigation, the efficacy and accuracy of *mip* gene based primers were tested in PCR for culture-negative samples.

Materals and Methods: The samples used in this investigation were previously reported as negative by means of conventional culture methods. DNA extraction was carried out by freezing-boiling method. The small fragment of mip gene (of *L. pneumophila*) was used for the primer set (LEG 1 and LEG-2) in PCR, and also for specific probe LEG-3 in southern blot.

Results: From 32 culture-negative samples subjected to these primer sets, *L. pneumophila* DNA was detected in 6 samples. System accuracy was then checked by dot blot hybridization.

Conclusion: The results of this investigation indicated that the PCR method, with suitable primer set and probe, detects the viable but non-culturable Legionella in clinical and environmental samples.

Keywords: Molecular detection, hybridization, dot blot , *Legionella pneumophila*

Introduction

Legionellae are ubiquitous environmental organisms which can infect humans, and in some cases, cause a life threatening pneumonia called "legionairs´ disease" or "pontiac" fever, commonly known as legionellosis (1). The relation of nearly 39 species and more than 60 serogroup of Legionellaceae has been described with human infections (2). Legionellae are facultative intracellular bacteria wich occur in different aquatic environments, where they could easily proliferate and infect human beings (3). Aerosol generators, such as water coolers which work with contaminated water, have been reported as the most important source of human infections (4). Other infection sources are ventilators, mineral springs, cold/ hot water distributing pipes in buildings and bath waters (5). Legionnaires´ disease was reported in special groups such as drug abusers, smokers, heavy drinkers and elders (6, 7). The identification of infection sources is often problematic due to the contamination of clinical and environmental samples with other micro-organisms (8). The culture method encountered some shortcomings, although it is still a golden standard (9) with sensitivity of 65%-80% (10). The viable but non-culturable *legionellae* is known as the most serious difficulty, as reported from clinical and environmental specimens (11). The application of nucleic acid based diagnostic method, like PCR, is used for clinical and epidemiological investigation, but it still faces some technical shortcomings which limit its use in routine laboratories. In polymerase chain reaction (PCR) technique, the small fragment of target DNA will duplicate in a logarithmic manner in a laboratory tube (12). This method should be an ideal alternative for detecting organisms not identified by the culture method. Different Primers and probe have been designed for legionellae detection (13). Stransberg et al. used a cloned fragment of *L. pneumophila* as the target sequence (14), while Mahbobani et al. designed two sets of primers (13). The PCR method has been optimized only for small portion of pathogens microorganisms. In the present investigation, suitable primer set and probe were used to test system sensitivity for culture negative water samples. The samples, which were assumed to be completely negative for legionella, contained very small numbers of organisms or viable but non-culturable legionella.

Materials and Methods

Water samples were provided by previously described methods (15). Briefly, the samples were first filtered through 0.22 μm membrane filter and the filter residual was then resuspended in 50 ml of the original filtrate. The concentrated samples were cultured on enriched buffered charcoal yeast extract (BCYE) agar with ferric pyrophosphate and L-cysteine (15). Glycine, vancomycin, polymixine and cyclohexamide were added to the medium to make it selective when tried with environmental samples (2). After incubation (two weeks at 37°c with 5% CO_2), all samples were put in culture negative and culture positive groups, the first group was subjected to further investigation.

One milliliter of each original sample was taken from the 50 ml water sample filtrate and placed in a 1.5 ml ependorf tube. DNA extraction was carried out according to previously published methods (8). Briefly, the samples were subjected to 3X freezing-boiling in liquid nitrogen (5 minutes each). The culture positive samples were also subjected to DNA extraction in the same way. The cell debris was separated and removed by centrifugation. The samples were then centrifuged at 10.000g for 10 minutes at room temperature. The supernatant was taken out, and 20 μl of each tube residue was subjected to PCR experiments.

Bacterial DNA was amplified by a thermocycler utilizing two 19 mer primers; LEG 1 (5´ -GTC ATG AGG AAT CTC GCT G-3´) and LEG 2 (5´-CTG GCT TCT TCC AGC TTC A-3´) specific for *L. pneumophila* chromosomal DNA sequence of unknown function (16).

The legionella oligonucleotides (LEG 1, LEG 2 and LEG 3) were prepared as a solution in ammonium hydroxide and purified with the ethanol percipitation method, as described by the supplier. Briefly, the suspensions containing oligonucleotides were placed in an oven for about 30 minutes and allowed to dry, then 200 μl of pre-cooled sodium acetate 0.3M was added to each sample and mixed well and, then

600 µl absolute ethanol was added and mixed well. The solution of oligonucleotides was kept at 70°C for about 15 min. The samples were centrifuged at 10.000g for 10 minutes at +4°C. The supernatant was removed by a sterile glass pasteure pipette, then the pellet was mixed with 1ml of pre-cooled absolute ethanol, and kept at 70°C for 15 min. They were dried in an oven and stored at -20°C.

Thirty five cycles of amplification were done, each cycle compromised of denaturation for 1 min at 93°C, annealing for 1 min at 55°C, and extension for 1.5 min at 74°C, this was followed by a final 7 min incubation at 74°C to maximized extension of the amplified products. The PCR reagents were Gene Amp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) and were used as recommended by the manufacturer. The controls used, were primers incubated with sterile and non-pyrogenic water. Gene Amp positive control was used as supplied, and *L. pneumophila* serogroup 1 positive controls were prepared as for the experimental samples.

Hybridization with a radiolabeled probe is the most sentitive method to detect specific DNA sequences. Thus, dot blots of the amplified products were prepared and probed with a specific 25-mer radio-labeled oligodeoxynucleotide LEG 3, (5'-GTC CGT TAT GGG GTA TTG ATC ACC A-3') and end labeled with y[^{32}P] dATP by polynucleotide kinase. The samples for bloting were removed from the reaction tubes after PCR amplification and boiled for 10 min. Then 30 µl was spotted onto a nitrocellulose membrane which was then baked under vacuum at 80°C for 2h, then was followed by incubation in a prehybridization buffer of 6x SSC, 5x Denhardt solution, and 0.1% SDS (16). The controls were pyrogen-free water and *L.pneumophila* (S1) as before.

Results

This investigation was carried out in order to test the primer designated for *L. pneumophila* chromosomal DNA. Hospital acquired pneumonitis are caused by *L. pneumophila* infections, which was the most prominent legionellac spp. Some predisposing factors are involved in infection prevalence within different societies. It would be essential to monitor various sporadic rocus of disease in the society in order to get the disease under control. As complementary to the culture method, the PCR technique seemed to work properly. From the 32 culture negative samples subjected to DNA amplification, *L. pneumophila* was detected in 6 samples. No band was seen in most (nearly 85%) negative samples (Fig 1).

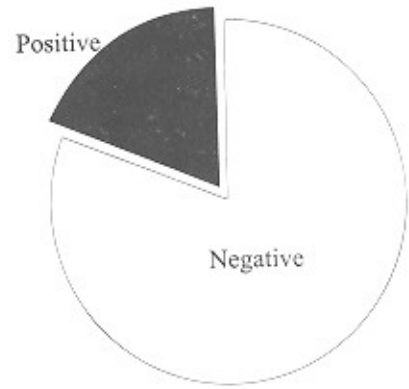

Fig 1: Number of positive samples by PCR technique.

Amplification products (0.8 kb) characteristics of those expected from *L. pneumophila* (Fig 2), were obtained from approximately 15% of the culture negative samples.

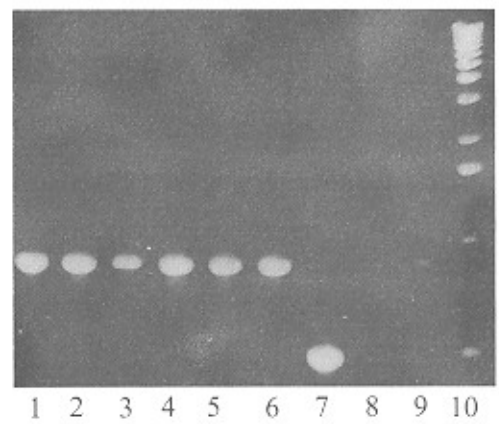

Fig 2: PCR products on agarose gel electrophoresis . 1-6: various samples, 7: amply kit gene, 8: distilled water, 9: *L. pneumophila* control positive, 10: 1 kb DNA ladder.

In each positive sample, the PCR product was confirmed as *L. pneumophila* DNA by hybridization using dot blot with a specific oligonucleotide probe for *L. pneumophila* (Fig 3).

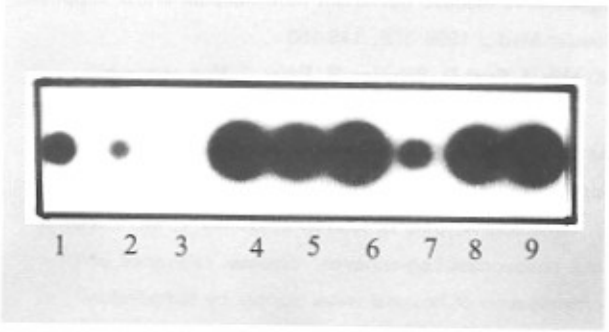

Fig 3: Dot blot of probed PCR products. *L. pneumophila* DNA labled with 32P. 1: *L. pneumophila* 1 (control), 2: *L. pneumophila* 1 (positive control), 3: distilled water, 4-9: samples.

Discussion

A considerable percentage of hospital acquired pneumonitis was caused by *L. pneumophila* (especially S1) as the most prevalence Legionellae in hospital establishments (17). Some predisposing factors such as immunity disorders are involved in incidence of legionellosis among vulnerable populations. In order to ease disease monitoring in the society and medical establishments, the culture method limitations should be removed by complimentary methods. The potable and non-potable water sources should be monitored properly (18). Reliable and rapid methods are necessary for disease diagnosis, epidemiological studies and also for preventative measures. The culture method for legionella, currently assumed to be applied as gold standard, has good specificity but the sensitivity is not enough to meet laboratory requirements (19). The main problem for field study of legionella by the culture method is the fastidious nature of the organism which takes too much time to grow up (1). Many researchers faced serious problems when growing up the organism or inoculating guinea pigs by suspected specimens. The viable but non-culturable legionellae are the major reasons for culture negative phenomenons in bacteriological laboratories (11, 20).

Amplification of specific DNA sequences by the polymerase chain reaction in association with the use of probe has been used for detecting legionella in environmental water samples (21). The primer set and probe used in this study was shown to be specific for legionella at species level (16). The number of thermocycler cycles were reduced to 35 cycles in order to obtain both good sensitivity and specificity from the system (13, 14, 22). The primer system and probe used in this study proved to have a good specificity for *L. pneumophila* , therefore, different serotype of the legionnaire's-disease bacterium would be detected by the system in clinical and field studies (13). Although, the sample size in the present study were not enough to prove PCR sensitivity for *L. pneumophila*, but bacterial DNA was detected in approximately 15% of the culture-negative samples. The primer set sensitivity for *L. pneumophila* was reported to be nearly 35 c.f.u. (10). The main message of this work is assumed for culture negative samples which are usually encountered both in clinical and environmental studies. This problem was removed partially by using two methods side by side. Different scientists recommended the reference laboratory to use PCR and the culture method for legionella when working with significant samples.

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