Research of Legionellla Pneumophila and Flora in the Circuit Contaminating Water Hotel Tunisian: Method by PCR and Sequencing

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Abstract:

***Aim** :*

In our study, we tried to search for Legionella pneumophila and flora who contamine water circuits Hotels Tunisians, using PCR and sequencing.

Material and methods :

This study was performed on 1145 samples from various water circuits between 01/08/2011 and November 2011 as well as those which were collected between 01/01/2011 and April 2011, from Tunisian Hotel units . We thus selected on BCYE alpha medium with L-cysteine, 31 strains.

Results :

45.2 % of Legionella type including: 16.13 % are Legionella pneumophila, 9.68 % are Legionella dumoffii, 6.45 % are Legionella anisa and 12.9 % are Legionella parisiensis. 41.9 % of the genus Bacillus, including: 22.58 % are Bacillus stratosphericus, 9.68 % are Bacillus subtilis, 9.68 % are Bacillus pumilus. 12.9 % are Staphylococcus haemolyticus.

Conclusion :

We conducted a phylogenetic tree that gave us exactly the percentages of contaminating bacteria in the water circuits. The PCR test 16 s, shows that we have different types of bacteria with Legionella pneumophila. Our study demonstrate also, that Legionella pneumophila and the others bacteria found in cold and many hot water circuits.

These methods are therefore, in our days, the most reliable to detect a maximum of pathogens.

Key words : Legionella pneumophila, Contaminating bacteria, Water circuit Hotel, PCR Method, sequencing.

1.Introduction :

Legionella pneumophila is a bacterium hydrotellurique, pathogen, causing pneumonia caused by inhalation of aerosols from water sources, such as distribution systems, showers, cooling towers and aéroréfrigérantes. Legionella pneumophila is responsible for 90% of cases of legionellosis. On the other hand, there may be seen in the water circuits of other types of contamination by pathogenic bacteria or various other ubiquitous. [1- 2- 3, 4].

The purpose of this study is to investigate and identify by PCR and sequencing, other than *Legionella pneumophila* bacteria to allow the total eradication of these.

2.Materials and methods :

2.1-strains

31 strains of 1145 samples from hot and cold water circuits (hot and cold water, a flexible ball room, a flexible bath room, hot water boiler return) were collected from 'Hotels Tunisia during the period from August 2011 to November 2011 and during the period from January 2011 to April 2011.

2.2-Methods

The 31 strains were isolated from samples of culture medium "buffer charcoal yeast extract" (BCYE). These strains are the result of a first transplant performed at Lab 21 on various samples of water from different sources (flexible bath, thermal pool, hot flexible ball, shower head, hot water boiler start 60 °C, hot water return 60 °C heating, cold water shower head) to ensure the various contamination by *Legionella pneumophila* and other germs. For puridfication of the strains, a second transplant was performed at the Laboratory of the Faculty of Sciences of Tunis, having been preceded by isolation and incubation for 4 to 10 days to get completely pure strains on Petri dishes.

We then proceed to extraction of the DNA by phenol chloroform.

16 s the PCR (Polymerase Chain Reaction) is subsequently used the reaction mixture and the concentration of reactants is shown in the table n°1. The PCR technique 16 s, is based on three steps:
- Denaturation of DNA to obtain single-stranded templates
- Hybridization comprising merely initiate and replication of the sequence to be amplified using specific oligonucleotide primers.
- Elongation of performing the polymerization reaction of the complementary strand. At the end of each cycle, the products are in the form of double stranded DNA.

Electrophoretic analysis was carried out thereafter.

It also makes the sequencing of determining the order of nucleotides on a fragment of rDNA 16 s. The sequencing is done according to the Sanger method, given in figure 2. [5- 6,-7- 8- 9, 10, 11].

The identification of strains, based on the sequences of the 16 s rRNA, is performed using the BLASTn program. The comparison is made with respect ausx reference strains available in the database accessible on the Web, NATIONAL CENTER FOR BIOTECHNOLOGY NCB INFORMATION. (http :/// www.ncbi.nlm.nih.go and also by the utilities ribosomal Data Base Project: RDP (http://rdp.cme.msu.edu).

3. Résults :

We recall that the 31 samples which we have already mentioned in the molecular underwent different treatments followed by incubation and subculturing of colonies typical for bacteria intensive L-cysteine and highlighting *Legionella*. To be safe on our results, we then thought of a confirmatory test by PCR 16S, followed by sequencing (Figures 1, 2, 3, 4, 5 and 6).

As shown in the tree above, the sequencing of 16S fragments covering the diversity of strains established for phylogenetic affiliation of the different isolates, allowed resemble the strains obtained two phyla: *Phylum Firmicutes*, class *Bacilli*, order and genus *Staphylococcus Bicillales*
-Proteobacteria phylum class *Gammaproteobacteria*, order and genus *Legionella Legionellales*. They are divided into three families (family *Bacillaceae*, the family of *Staphylococcaceae*, family of *Legionellaceae*) where we see that there is not only the genus *Legionella* but two other genera with percentages: 45;2 % of *Legionella* type including: 16.13 % are *Legionella pneumophila*, 9.68 % are *Legionella dumoffii*, 6.45 % are *Legionella anisa* and 12.9 % are *Legionella parisiensis*. 41.9 % of the genus *Bacillus*, including: 22.58 % are *Bacillus stratosphericus*, 9.68 % are *Bacillus subtilis* and 9.68 % are *Bacillus pumilus*. 12.9 % are *Staphylococcus haemolyticus*.

The curve (figure 6), demonstrate what we found for sequençage of *Legionella pneumophila*.

The table 2 demonstrate that *Legionella pneumophila* contaminates the hot water, the flexible with a ball room, hot water tank bottom No. 1 second jet, bottom ball No. 2 second jet. *Legionella dumoffi* contaminates the hot water tank down No. 1 second jet, bottom ball No. 2 second jet and flexible bathroom. *Legionella anisa* contaminates the hot water flexible ball second jet. *Legionella parisiensis* contaminates the hot water tank down No. 1 second jet, flexible ball second jet, back boiler. *Staphylococcus haemolyticus* contaminates cold water, hot water bottom flask No. 1 second jet; flexible bath second jet, and of the flexible bathroom. Various *Bacillus* contaminates the cold water and hot water from different sources.

4.Discussion

If we are interested in the *Legionella* genus, three species other than *Legionella pneumophila* were present what is acceptable, since they have almost all the same biochemical and microbiological characteristics of pathogenicity but less severe.

Legionella anisa is one of the most common species of *Legionella* other than *Legionella pneumophila* in the environment and can be acquired in the hospital in rare cases. We found that *L. anisa* may mask water contamination by *L. pneumophila*, which suggests that there is a risk of infection with *L. pneumophila* in immunocompromised patients, if the water is contaminated. It appears that the *Legionella anisa* acts as an indicator in the water. His presence automatically requires the presence of *Legionella pneumophila* [1-2- 3, 4]. (Figure 4). In our study, *Legionella pneumophila*, *Legionella dumoffi*, *Legionella anisa* and *Legionella parisiensis* contamine only the hot water circuits. *Staphylococcus* contamines cold water and hot water circuit. Diverses *Bacillus*, contamine cold water and hot water circuits (table 2).

In the study by Perola O. *et a*l [12], after a nosocomial outbreak caused by *Legionella pneumophila serogroup 5*, the hospital water distribution system, which was found to be colonized by *L. pneumophila serogroup 5 and 6*, was decontaminated by the superheat and flush method and by installing an additional heat-shock unit in one of the hot water circuits. The unit exposed the circulated water to a temperature of 80 degrees C.

Legionella are prevalent in human-made water systems and cause legionellosis and a severe form of pneumonia in humans. [4- 13]..Thirteen chromosomal restriction patterns determined by Pulsed field gel electrophoresis were detected [3]. We report a rapid detection method for viable *Legionella* that combines

ethidium monoazide (EMA) with quantitative real-time PCR (qPCR) and apply this method to detect *Legionella* in a large number of water samples from different sources [14] (Figures 1, 2, 3, 4, 6 and 6). These results are consistent with those of our study. A study by Zhang Q. *et al*. [15] was conducted to understand the contamination by *Legionella* in water samples from various locations in cold Wuxi City and this study reveals the same time, the biological characteristics of strains of *Legionella*. The positive proportion of *Legionella* was 39.3 % (22/56) among all sampling sites. This, joined about our results are 45;2 % of all types of *Legionella.* The *Legionella* contamination existed in cooling water samples from different places in Wuxi City same our results in Tunis. *Legionella* strains isolated showed high genetic variation. Some *Legionella* strains had vigorous intracellular growth ability [15] (Figure 5). Of *Legionella pneumophila* were even found in the wash water dental offices dentists [16- 17, 18].

Our results and the other study, support the opinion that PCR cannot be a substitute for the isolation of *Legionella* by culture , it could be regarded as an useful complementary method same we made in our research [19] (Figure 2).

Staphylococcus aureus, Pseudomonas aeruginosa and *Legionella pneumophila* have been detected in indoor air and linked to human infection. The study by Bandyopadhyay P. *et al.* [20], demonstrate that *Legionella pneumophila* , the causative agent of "Legionnaires disease", is a ubiquitous freshwater bacterium whose virulence phenotype require a type IV secretion system (T4SS). The study by Chen Ml. *et al*. [21] demonstrate that on 131 strains isolated from the 16 cooling towers, it includes *L. pneumophila, L. bozemanae, L. micdadei* and *L. anisa*]. In the study by Preston Ma *et a*l [22], it's demonstrated an epidemiologic subtyping of *Escherichia coli serogroup O157* strains isolated in Ontario (Canada) by phage typing and pulsed-field gel electrophoresis. In the study by Us E. *et al*. [23], there's an investigation of *Salmonella serotype enteritidis* isolates by plasmid profile analysis and pulsed field gel electrophoresis.. In our investigation, there's another types of bacteria same diverses *Staphylococcus* and *Bacillus*. *Legionella pneumophila* is detected in many studies by PCR same our study. The selection of the method to be used depend on technical (rapid, low-time-consuming, easy to perform and to performand to interpret, reproducible and discriminatory) and economical (low cost) factors [24].

Practically, the same study was conducted in 9 provinces of China where *Legionella pneumophila* was identified in water with various methods that we studied in our research [25]. In our study, we investigated the *Legionella pneumophila* in water systems Tunisians Hotels and pensions we find only this species. It turned out that in parallel, we found other pathogenic species despite the use of selective methods to isolate *Legionella pneumophila*..

These results demonstrate that one can find in parallel to other *Legionella* species pathogens through the performance of PCR and sequencing that has been used. Our study demonstrate also, that Legionella pneumophila and the others bacteria found in cold and many hot water circuits.

Références

1. Gea-Izquierdo E., Mezones-Holguin E. and Haro-Garcia L. Actions for legionellosis prevention and control : a challenge for public health in Spain. Rev. Peru Med. Exp. Salud. Publica. 2012; 29 : 272-6.
2. Amemura-Maekawa *et al*. Distribution of monoclonal antibody subgroups and sequence- based types among *Legionella pneumophila serogroup 1* isolates derived from cooling tower water, bathwater, and soil in Japan. Appl.Environ. Microbiol. 2012;78 : 4263- 70.
3. Weber Ss. *et al.* Identification of Protective B Cell Antigens of *Legionella pneumophila*. J. Immunol. 2012; 189 : 841- 9.
4. Jamillouw Y., Jarraud S., Lina G., Etienne J. and Ader F. *Legionella*, Legionnaires' disease. Med. Sci. (Paris). 2012; 28: 639- 45.
5. Mccoy Wf. *et al*. Inaccuracy in *Legionella* tests of building water systems due to sample holding time. Water Res. 2012; 46 : 3497-506.
6. Chien M. *et al.*The genomic sequence of the accidental pathogen Legionella Pneumophila. Science. 2004; 305 : 1966- 8.
7. Van Belkum *et a*l. Assessmentof resolution and intercenter reproducibility of results of genotyping *Staphylococcus aureus* by pulsed-field gel electrophoresis of Smal macrorestriction fragments : a multicenter study. J. Clin. MIcrobiol. 1998; 36 : 1653-9.
8. Pruckler J.M. *et al*. Comparison of Legionella pneumophila isolates by arbitrarily primed PCR and pulsed-field gel electrophoresis : analysis from seven epidemic investigations. J. Clin.Microbiol.

1995; 33 : 2872-75.
9. Chang B, Amemura-Maekawa J. and Watanabe H. An improved protocol for the preparation and restriction enzyme digestion of pulsed-field gel electrophoresis agarose plugs for the analysis of *Legionella* isolates. Jpn. J. Infect.Dis.2009; 62 : 54-6.
10. Lee Hk., Kang Yk. and Yu Jy. Genomic diversity of *Legionella pneumophila serogroup 1* from environmental water sources and clinical specimens using pulsed-field gel electrophoresis (PFGE) from 1985 to 2007, Korea. J. Microbiol. 2010; 48 : 547- 53.
11. Chen Nt., Chang Cw. and Wu Jd. Evaluation of DNA isolation procedures for detecting and quantifying environmental *Legionella* by real-time quantitative PCR. Water Sci. Technol. 2012; 65 : 989- 97.
12. Biurrun A; *et al.* Treatment of a *Legionella pneumophila* colonized water distribution system using copper-silver ionization and continuous chlorination. Infect. Control. Hosp. Epidemiol. . 1999; 20 : 426-8.
13. Dronina Iug *et al*. Serologic characteristic of *Legionella pneumophila* strains isolated from potentially dangerous water systems in Russian Federation in 2007-2011. Zh. Mikrobiol.Epidemiol. Immunobiol.. 2012; 2 : 23-8.
14. Qin T. *et al*. Application of EMA-qPCR as a complementary tool for the detection and monitoring of *Legionella* in different water systems. World . Microbiol.Biotechnol. 2012; 28 : 1881-90.
15. Zhang Q. *et al*. Contamination status and molecular biological characteristics of *Legionella* in cooling water samples from different places in Wuxi city. Zhonghua Yu Fang Yi Xue Za Zhi. 2012; 46: 264- 8.
16. Marranzo M. *et al*. Evaluation of biological indicators of hygienic quality in modern dental clinics with a high perceived quality in Catania (Italy). Ig. Sanita. Pubbl. 2010; 66 : 757-66.
17. Palenik Cj. *Legionella pneumonia* and dental unit waterlines. Dent. Update. 2012; 39: 237-8.
18. Ricci Ml. *et al*. Pneumonia associated with a dental unit waterline. Lancet. 2012: 379: 684.
19. Stojek Nm. *et al*. Efficacy of the detection of *Legionella* in hot and cold water samples by culture and PCR . II. Examination of native samples from various sources. Ann. Agric. Environ. Med. 2012; 19: 295- 8.
20. Bandyopadhyay P. *et al.* Implication of proteins containing Tetratricopeptide Repeats in cond itional virulence phenotypes of *Legionella pneumophila*. J. Bacteriol. 2012; 194: 3579-88.
21. Chen Ml. *et al*. Genotypic variability and persistence of *Legionella* pulsed-field gel electrophoresis patterns in 16 cooling towers in Shanghai, China. Zhonghua Liu Xing Bing Xue ZA Zhi. 2010; 31: 771-5.
22. Preston Ma, Johnson W., Khakhria R. and Borczyk A. Epidemiologic subtyping of *Escherichia coli serogroup O157* strains isolated in Ontario by phage typing and pulsed-field gel electrophoresis. J. Clin. Microbiol. 2000; 38: 2366- 8.
23. Us E. *et al*. Investigation of *Salmonella serotype enteritidis* isolates by plasmid profile analysis and pulsed field gel electrophoresis. Mikrobiyol. Bul. 2011; 45: 210-27.
24. Paveenkittipom W. Dejsirilert S. and Kalambaheti T. Genetic speciation of environmental *Legionella* isolates in Thailand. Infect. Genet. Evol. 2012; 12: 1368-76.
25. Zhu Bq *et al.* Sequence –based typing of 82 strains of serotype I *Legionella pneumophila* isolated from 9 provinces in China. Zhonghua Yu Fang Yi Xue Za Zhi. 2011; 45: 890-4.

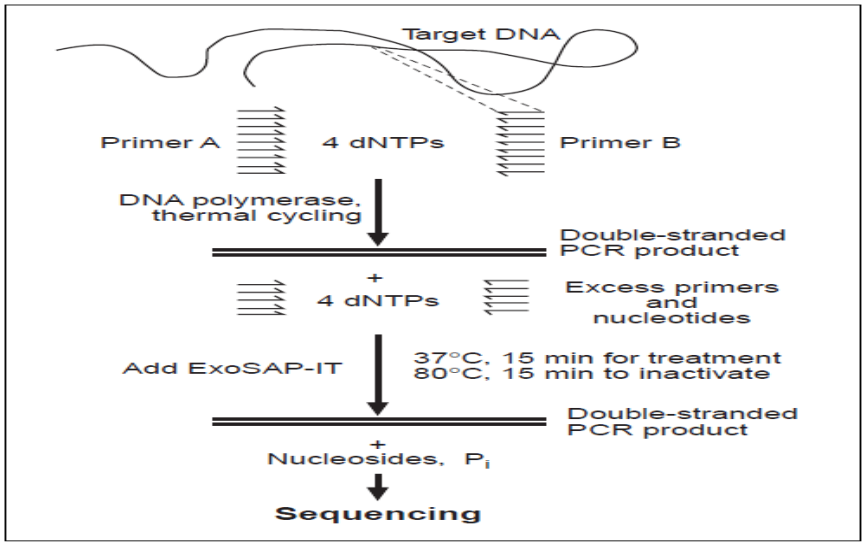

Figure 1: Purification enzymatic

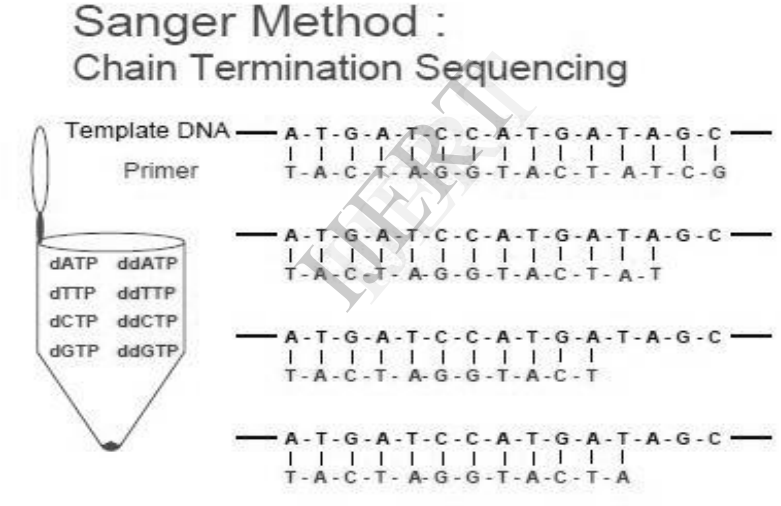

Figure 2: The DNA labeling

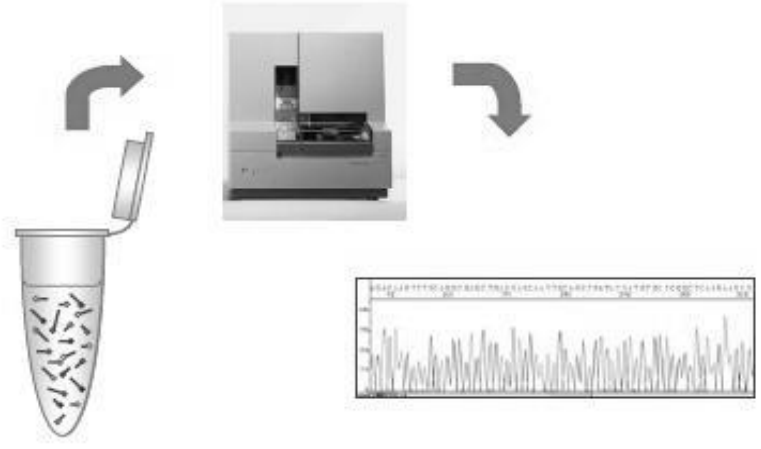

Figure 3 : Injection sequencers

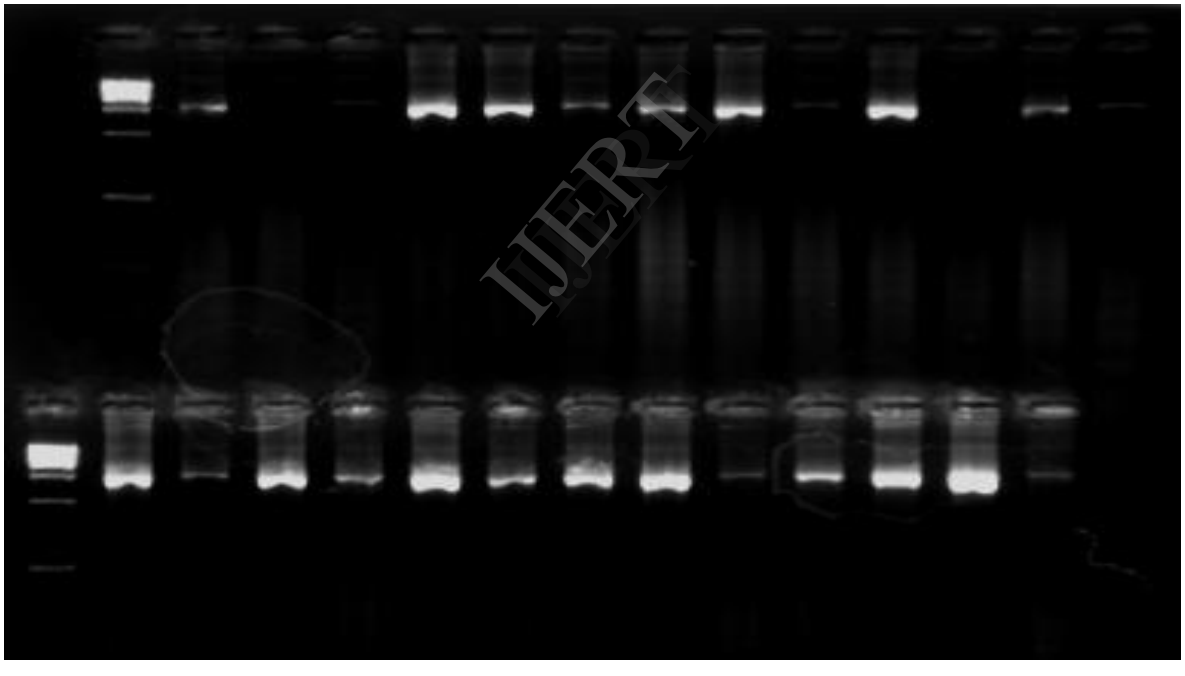

Figure 4: Result of PCR: Visualization of DNA in black and white

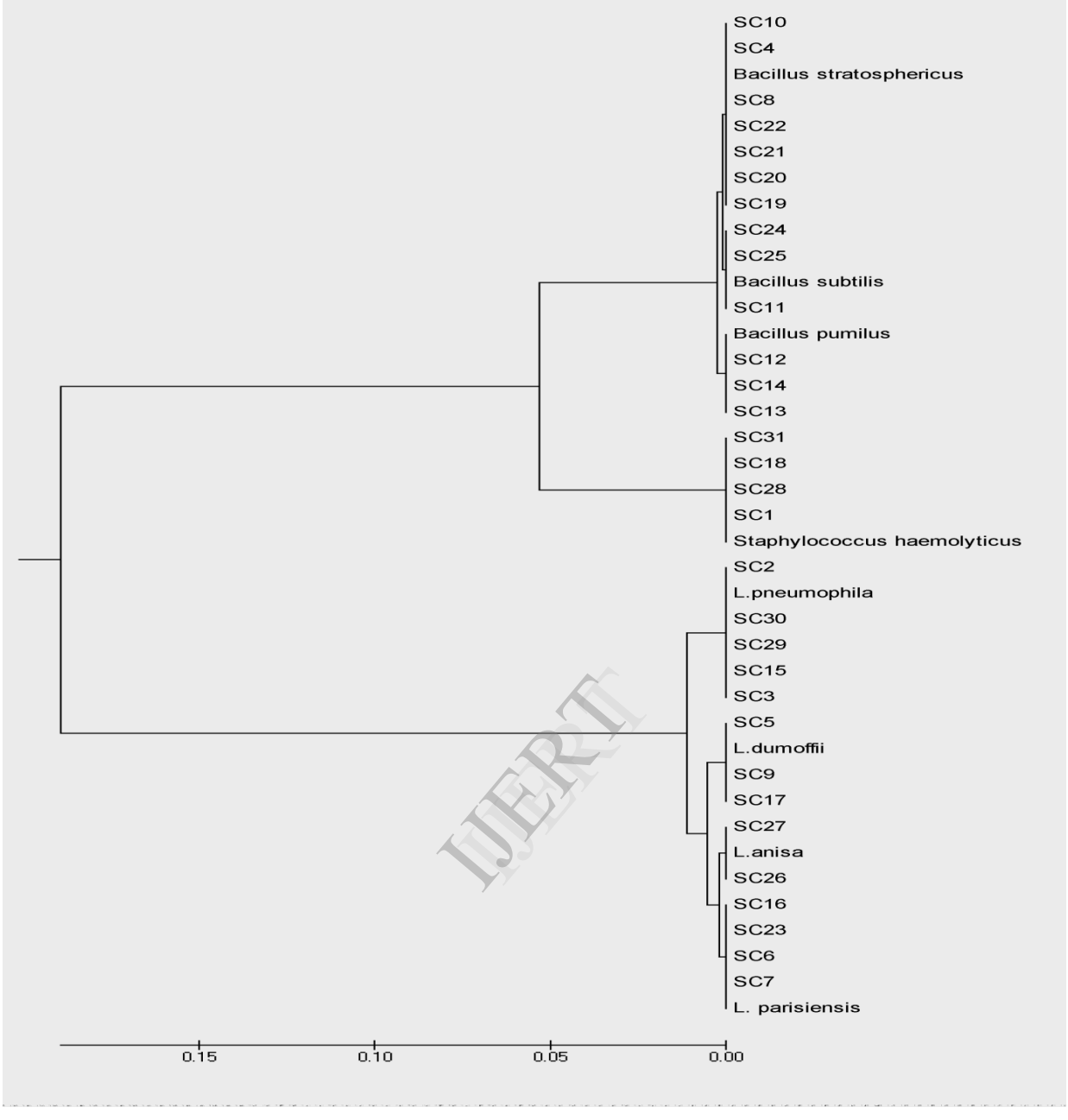

Figure 5: Phylogenetic tree of 31 strains with reference strains

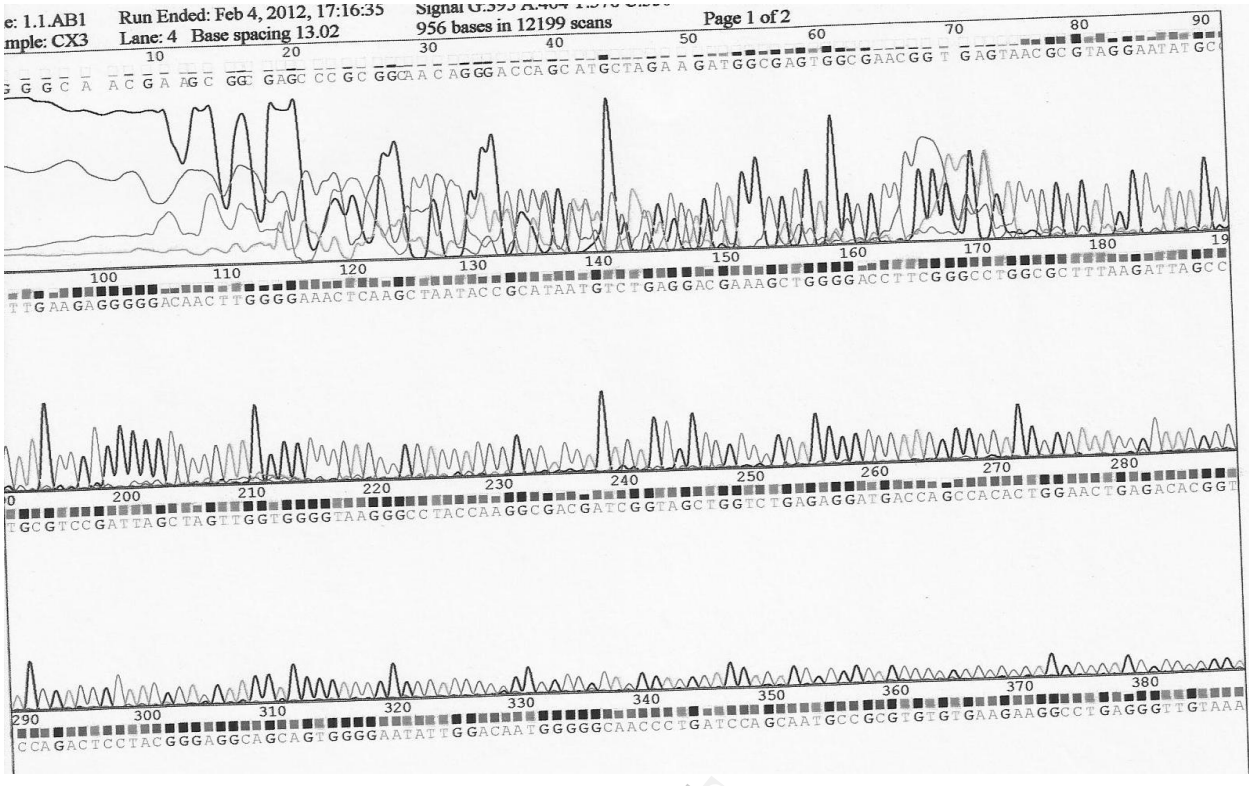

Figure 6 : Curves and sequencing of *Legionella pneumophila*

Components of the reaction mixture	Amplification 16S	
Enzyme buffer	10X	4µl
MgCI2	25m M	
dNTP	25mM	0,2µl
Boot F	25µM	0,3µl
Boot R	25µM	0,3µl
Taq polymérase	1U	0,22µl
H_2O	18.98µl	18.98µl
cDNA	250ng/µl	1µl

Table 1: Components of the reaction mixture of PCR 16S.

1) Cold water	SC1, SC13
2) Hot water	SC2, SCA, SC10, SC11, SC12, SC14
2.1 Flexible of ball room:	SC3, SC15, SC21, SC22
2.2 Back boiler room:	SC6, SC7
2.3 Restoration boiler room, second jet:	SC8
2.4 Lower ball No. 1, second jet:	SC16, SC17, SC28
2.5 Lower ball No. 2, second jet:	SC29, SC30;
2.6 Flexible bathroom, second jet:	SC18, SC19, SC20, SC24, SC25
2.7 Flexible ball, second jet:	SC23 SC31, SC26, SC27
2.8 Flexible bathroom:	SC5; SC9

Table 2 : Different sources of various samples