ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine 2013, Vol 20, No 4, 649–652
www.aaem.pl

Legionella pneumophilla bacteria in a thermal saline bath

Maciej Walczak[1], Arkadiusz Krawiec[2], Elżbieta Lalke-Porczyk[1]

[1] *Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University, Toruń, Poland*
[2] *Department of Geology and Hydrogeology, Nicolaus Copernicus University, Toruń, Poland*

Walczak M, Krawiec A, Lalke-Porczyk E. *Legionella pneumophilla* bacteria in a thermal saline bath. Ann Agric Environ Med. 2013; 20(4): 649–652.

Abstract

Objective: The study was aimed at determining whether *Legionella pneumophila* bacteria can be found in thermal saline waters used in balneotherapy.

Methods: Water samples were collected from three thermal saline baths, supplied by thermal saline waters (type Cl – Na). The total number of bacteria was determined in the direct microscopic count under a fluorescence microscope The numbers of bacteria belonging to different phylogenetic groups (Eubacteria, *Legionella sp.* and *Legionella pneumophila*) were determined with the use of a molecular FISH method.

Results: The highest average total number of bacteria as well as the highest average number of Eubacteria in the entire research cycle was recorded in bath 1. Bacteria belonging to the *Legionella* genus along with *Legionella pneumophila* were identified in all water samples collected from each bath. Moreover, biofilm containing cells of *L. pneumophilla* was identified in the collected water samples.

Conclusion: The number of bacteria in water increases with the bath's age. The *Legionella pneumophila* can successfully develop not only in fresh water bodies but in thermal saline baths as well. Still, it is uncertain whether the commonly applied culture method, developed for freshwater bodies, is also suitable for thermal saline baths.

Key words

environment and public health, *Legionella pneumophila*, thermal bath

INTRODUCTION

First identified in 1976 *Legionella sp.* [1, 2], bacteria are one of the main groups of pathogenic bacteria transmitted via water [3] and belong to the gamma *Proteobacteria* [4]. Approximately half of 48 species of *Legionella* cause a disease called legionellosis (Legionnailers' disease), most notably *Legionella pneumophila*, responsible for more than 80% of all known cases [5]. Human infection by *Legionella pneumophila* usually results from inhaling aerosol – droplets of water which contain bacterial cells [6].

The natural habitat of *Legionella sp.* is the aquatic environment – they are present both in natural water bodies (lakes and rivers) and in artificial (anthropogenic) ones. Until 1993, when they were identified in the ocean, they had been considered incapable of surviving in saline environments. They were also recovered from compost, sewage, and soil [2]. *Legionella* bacteria move from natural to anthropogenic environments, finding particularly favourable conditions for development in hot water systems, where the temperature ranges from 30 °C to 40 °C [7].

Although the optimum temperature for the growth of *L. pneumophila* is 32 – 35 °C, they can survive in temperatures ranging from 0 °C to 70 °C. Due to the fact that another critical determinant of their development is oxygen concentration (they need good aerobic conditions), they are common in heated swimming pools, recreational facilities used in balneotherapy as well as in showers, fountains, and cooling towers.

Address for correspondence: Maciej Walczak, Nicolaus Copernicus University, Department of Environmental Microbiology and Biotechnology, Lwowska 1, 87-100 Toruń, Poland
e-mail: walczak@umk.pl

Received: 13 March 2012; accepted: 18 February 2013

L. pneumophila bacteria are known to thrive in biofilm, which forms at solid – liquid interfaces or at the liquid – air interface (floating biofilm, surface microlayer) [8].

The present study was aimed at determining whether *Legionella pneumophila* bacteria can be found in thermal saline waters used in balneotherapy.

MATERIALS AND METHOD

Sample collection. Water samples were collected from thermal saline baths, supplied by thermal saline waters (type Cl – Na), containing mainly iodides and iron, components with pharmacodynamic properties (Tab. 1). Found at great depths (700 – 1700 m), they are well-isolated from surface waters and appear to contain almost no organic compounds. The temperature of the water in the intake ranges from 32 °C to 40 °C. There is a continuous water flow from the intake into the pipes.

Water samples were collected from November 2010 to May 2011 (5 sampling cycles) from three thermal baths:

Bath 1 – water salinity – 5%, built over 80 years ago, bath 2 – water salinity 4%, built over 50 years ago, bath 3 – water salinity 1.5% – built 5 years ago. Baths 1 and 2 are used for balneotherapy, bath 3 is used for recreation only.

Each sampling operation involved collecting one litre of water into sterile glass bottles from each bath. At the same time, the following physicochemical parameters of water were measured: its temperature, redox potential, pH value (with the Elmetron pH-meter), and oxygen saturation (with the Hanna Instruments oximeter). The samples were then transported to the laboratory in 7 °C.

Table 1. Physical and chemical properties of thermal saline waters.

Components / Properties	Intake 1, water for bath 1 and 2		Intake 2, water for bath 3	
	[mg x dm^{-3}]	[% milivals]	[mg x dm^{-3}]	[% milivals]
Mineralisation	43,520.0	----	761,64.0	----
pH	6.9	----	6.2	----
Na^+	14,700.0	85.25	25,520.0	84.13
Ca^{2+}	1,322.6	8.79	2,404.8	9.10
Mg^{2+}	486.1	5.33	960.05	5.99
K^+	163.2	0.01	196.6	0.38
Fe^{2+}	1.25	0.01	10.5	0.03
Cl^-	26,233.0	98.93	46,085.0	98.90
SO_4^-	96.7	0.28	510.26	0.81
HCO_3^-	355.8	0.78	187.2	0.23
Br^-	7.4	0.01	98.0	0.14
J^-	2.1	0.00	3.5	0.00
S (II) H_2S+HS^-	0.90	----	nd	----
Temperature	28 °C	----	40.5°C	----

nd – no data

MICROBIOLOGICAL ANALYSIS

Total number of bacteria. The total number of bacteria was determined in the direct microscopic count under a fluorescence microscope. A required volume of water was filtered through a membrane filter with a pore size of 0.22 mm. Microbial cells captured on the surface of the filter were stained with 2% solution of 4,6- diamidino-2-phenylene (DAPI). After 2 minutes the staining solution was removed, the filter was rinsed in ethanol and distilled water, and dried. The subsequently prepared microscopic slides were viewed under a microscope at a magnification 1000 X. 20 different fields of view were analysed in a single test. The number of bacteria was determined with the use of Multi Scan programme.

Fluorescence in situ hybridization (FISH) method. The numbers of bacteria belonging to different phylogenetic groups (Eubacteria, *Legionella sp.* and *Legionella pneumophila*) were determined with the use of a molecular FISH method.

After the water samples were fixed with formamide, the water was filtered through polycarbonate membrane filters with a 0.22 µm pore size in order to capture particles bigger than 0.22 µm. The hybridization was performed according to Grimm et al. [9]. Bacterial cells retained on the surface of the membrane were hybridized using species-specific fluorescently labelled (dye CY3) *oligonucleotide probes EUB338, LEG705, LEGPNE1 (oligo pl.)*. The probes were suspended in the hybridization buffer consisting of formamide 40% [vol/vol]; NaCl 0.9 M, sodium dodecyl sulfate 0.01%, Tris/HCl, pH 7.6 20 mM). The final concentration of the probes was 30ng.

This solution was applied to the surface of the filter with captured cells. The filter was then placed for two hours in a hybridization chamber and ultrathermostat at 46 °C. After that, in order to remove the unbound probes, the filters were placed for 15 minutes in the washing buffer (the composition of the buffer: 20 mM Tris/HCl, pH 7.6, 0.01% sodium dodecyl sulfate, 5 mM EDTA-160 mM NaCl), rinsed with the distilled water and dried. Then the filters were covered with the mixture of immersion oil and Citifluor AF2 (Citifluor Ltd. London, United Kingdom). Fluorescence was detected using Olympus BX50 microscope, equipped for epifluorescence microscopy with a 50W mercury high pressure bulb and the appropriate filter set 00 and 10 and at a magnification 1000 X. Colour micrographs were taken with digital image processing (Olympus XC50) using the software package (Cell^B v. 3.1.). The number of bacteria in the investigated slides were evaluated using MultiScan Base programme.

STATISTICAL ANALYSES

Statistical analyses were done using program STATISTICA 6.0. Aanalysis of Variance (ANOVA) and correlation was the statistical methods used in calculations. The results were analyzed considering the occurrence of bacteria of the total number of bacteria and all investigated phylogenetic bacterial groups (Eubacteria, *Legionella sp.* and *Legionella pneumophilla*) in relation to the physicochemical parameters of water in the investigated baths and of the bath's age.

RESULTS

The temperature in the investigated baths ranged from 30°C to 36.5 °C (Tab. 2), thus providing optimum conditions for the growth of *Legionella pneumophila*. Slight fluctuations of pH values did not affect the growth of the studied microorganisms. However, oxygen saturation varied significantly; the biggest differences between consecutive measurements being recorded in thermal bath 1 (range 19.8 – 138%).

Table 2. Physicochemical parameters of water in the investigated baths.

Data of sampling	Bath 1			Bath 2			Bath 3		
	Temperature [°C]	pH	Oxygen [%]	Temperature [°C]	pH	Oxygen [%]	Temperature [°C]	pH	Oxygen [%]
11,2010	32.5	8.0	19.8	32.3	7.4	102.6	36.5	7.2	133.6
01,2011	32.3	7.3	138.0	32.4	7.4	119.0	36.1	7.3	91.7
02,2011	31.5	7.2	70.0	32.2	7.4	110.0	35.6	7.4	102.5
03,2011	30.7	7.5	107.3	32.2	7.4	102.0	32.5	7.4	85.0
05,2011	31.7	7.1	67.0	32.3	7.5	102.6	34.7	6.7	117.5

The highest average total number of bacteria as well as the highest average number of Eubacteria in the entire research cycle was recorded in bath 1 (164.08×10^6 and $98.45 \times 10^6 \times dm^{-3}$ respectively (Fig. 1)). In bath 2 the average total number of bacteria was $94.84 \times 10^6 \times dm^{-3}$ while the average number of Eubacteria was lower and amounted to $55.96 \times 10^6 \times dm^{-3}$ during the entire research cycle. The lowest average total number of bacteria was recorded in bath 3 ($60.08 \times 10^6 \times dm^{-3}$). The average number of Eubacteria in this bath was $39.54 \times 10^6 \times dm^{-3}$.

Statistical analysis revealed no significant relationships between physicochemical parameters of water in the investigated baths and both the total number of bacteria and the number of Eubacteria ($p<0.05$). In addition, no significant changes in the number of bacteria of the investigated group

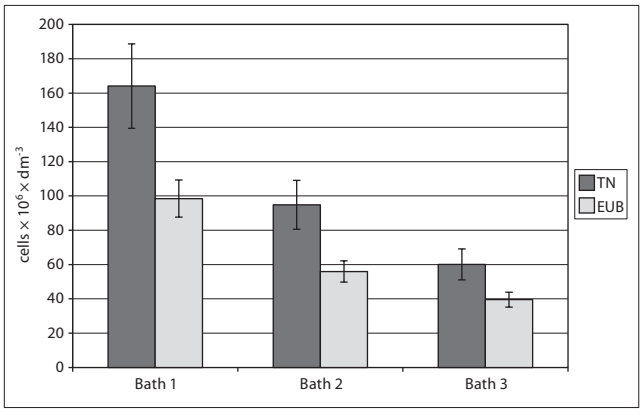

Figure 1. Total number of bacteria (TN) and number of Eubacteria (EUB) in collected water samples. Bars: Mean±SD.

were recorded in particular baths in consecutive samples. By contrast, the differences between the total number of bacteria, the total number of Eubacteria, number of bacteria *Legionella sp.* and *Legionella pneumophila* recorded in different baths are statistically important (p>0.05).

The figures reveal positive correlation (0.9) between the total number of bacteria and the number of Eubacteria. It was also established that the number of bacteria in water increases with the bath's age.

Bacteria belonging to the *Legionella* genus along with *Legionella pneumophila* were identified in all water samples collected from each bath (Fig. 2). Moreover, in the investigated microscopic slides *Legionella pneumophila* were also detected within the biofilm (Fig. 3).

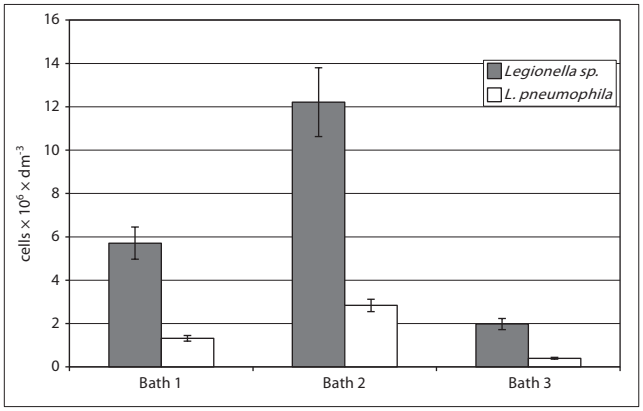

Figure 2. Number of *Legionella sp.* and *L. pneumophila* in the collected water samples. Bars: Mean±SD.

The highest number of bacteria belonging to the *Legionella* genus was recorded in bath 2 ($12.21 \times 10^6 \times dm^{-3}$ on average). In the water of this bath *Legionella sp.* constituted 21.8% of Eubacteria on average. Moreover, in bath 2 the biggest differences between the lowest and the highest numbers of these bacteria were recorded: the lowest being $1.24 \times 10^6 \times dm^{-3}$, and the highest being $32.47 \times 10^6 \times dm^{-3}$.

Furthermore, *Legionella pneumophila* bacteria were also the most abundant in bath 2, where their number ranged from $0.15 \times 10^6 \times dm^{-3}$ to $4.85 \times 10^6 \times dm^{-3}$ and amounted to $2.84 \times 10^6 \times dm^{-3}$ on average (Fig. 2). *L. pneumophila* constituted 5.07% of Eubacteria and 23.3% of *Legionella sp.* on average.

The lowest number of *Legionella sp.* ($1.98 \times 10^6 \times dm^{-3}$ on average) was observed in bath 3. The highest number of

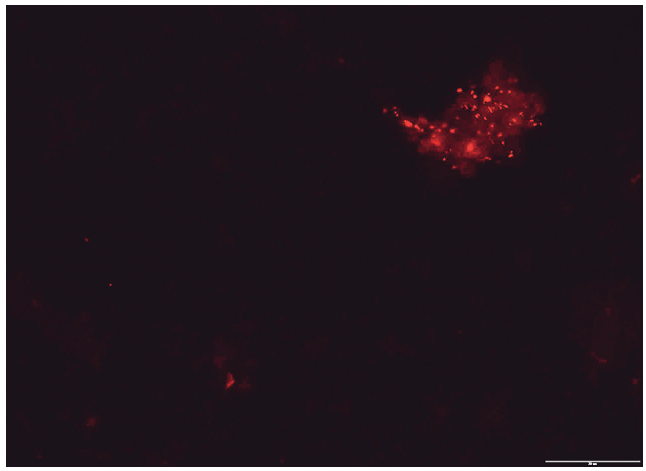

Figure 3. *Legionella pneumophila* – bacteria in the fragment of biofilm in investigated sample water from bath 1, bar = 20 µm.

Legionella sp. in bath 3 amounted to $3.36 \times 10^6 \times dm^{-3}$, while the lowest number amounted to $0.47 \times 10^6 \times dm^3$. Bacteria belonging to the *Legionella* genus constituted 5.0% of Eubacteria on average.

Again, *Legionella pneumophila* were the least abundant in bath 3. Their average number was $0.40 \times 10^6 \times dm^{-3}$, their lowest number was $0.07 \times 10^6 \times dm^3$, and their highest number was $1.12 \times 10^6 \times dm^{-3}$. *L. pneumophila* constituted 1.0% of Eubacteria and 20.2% of *Legionella sp.* on average.

In bath 1 the average number of bacteria belonging to the *Legionella* genus was $5.71 \times 10^6 \times dm^{-3}$, the lowest number was $1.36 \times 10^6 \times dm^{-3}$ and the highest number was $13.75 \times 10^6 \times dm^{-3}$. Bacteria belonging to the *Legionella sp.* genus constituted 5.79% of Eubacteria.

In bath 1 the number of *L. pneumophila* ranged from $0.36 \times 10^6 \times dm^{-3}$ to $3.11 \times 10^6 \times dm^{-3}$ while their average number was $2.84 \times 10^6 \times dm^{-3}$. *L. pneumophila* constituted 1.34% of Eubacteria and 23.1% of *Legionella sp.*

DISCUSSION

In the hitherto published research papers hardly any information is available on the distribution of *Legionella i*n thermal saline waters applied in balneotherapy. A majority of studies are aimed at identifying bacteria belonging to the family *Legionellaceae* in hot springs and in recreational facilities connected with the springs as well as in drinking and industrial (cooling systems, hydro-electric power stations) water supply systems. The results obtained during this investigation can be therefore regarded as original and innovative.

Taking into consideration the substantial salinity of the investigated thermal saline baths the estimated number of *Legionella pneumophila* seems to be large. However, Barbaree et al. [10] observed that *L. pneumophila* serogroups 4 and 5 display the highest resistance to chloride-sodium salt of all bacteria belonging to the genus *Legionella sp.* Besides, Palmera et al. [11] confirmed the presence of *Legionella sp.* in 50% of the collected samples of ocean water and the presence of *L. pneumophila* in 75% of the samples, which indicates a fairly high tolerance of this kind of bacteria to high concentration of NaCl. At the same time, as can be seen from the previous investigations, artificial ecosystems promote the

growth of these microorganisms, which appear to be more abundant in artificial than in natural water bodies [7, 12].

It should be noted, however, that all attempts to culture *Legionella sp.* from ocean water proved unsuccessful, which may indicate that only non-culturable cells of these microorganisms are present in saline waters. What is more, *Legionella* growth is the most rapid within the existing biofilm [6]. As was indicated before fragments of biofilm were found in the water samples collected from the investigated thermal saline baths. Since biofilm is known to possess some properties protecting bacterial cells found deep within this formation, professional antiseptics have little or no effect on them. It may be assumed that biofilm at the interface effectively protects *Legionella* against elevated concentrations of salt.

In this investigation the number of *Legionella* was within the range 10^3 to 10^8 CFU/dm^3, which corresponds to the range observed in the natural environment [13]. The results of the investigations conducted in hospitals and spas in Poland, (including those focused on examining water from thermal saline baths) show that cultured forms of *L. pneumophila* were found in 78.7% of the samples [13]. In addition, in a majority of positive tests (71.7% of the samples) the numbers of *L. pneumophila* were similar to the results obtained in this investigation.

In the investigations based on the real-time PCR method the presence of *Legionella sp.* and *Legionella pneumophila* was confirmed in all samples collected from anthropogenic water bodies [14]. Huang et al. [15] also found *Legionella sp.* in recreational areas centred around hot springs (well-developed infrastructure of public swimming pools, steam rooms, spas and bubble baths) in Taiwan. 20 samples out of 72 collected in the research cycle contained *Legionella sp.*

According to Exner and Hartemann [16] the outbreak of diseases caused by *Legionella pneumophila* seems unlikely as long as the bacteria do not exceed the number of$<10^5$ – 10^6 cfu/dm^3. It can therefore be concluded that thermal saline waters examined in this study are relatively safe for humans as the number of *L. pneumophila* ranged from 0.47 to $4.85 \times 10^6 \times dm^{-3}$ However, the number of bacterial cells identified with FISH method is certainly much higher than the number of culturable bacterial cells marked CFU.

It should also be pointed out that there have been cases of legionellosis caused by *Legionella* in aquatic systems when their number was higher than 10^4 cfu/dm^3 [17, 18].

The results obtained during this investigation show that *Legionella pneumophila* can successfully develop not only in fresh water bodies but in thermal saline baths as well. Due to the serious threat posed by these bacteria, regular monitoring appears to be a necessary measure. Still, it is uncertain whether the commonly applied culture method, developed for freshwater bodies, is also suitable for thermal saline baths.

Acknowlegment
This research has been carried out thanks to a State Committee for Scientific Research, grant NR: 3878/B/P01/2010/38

REFERENCES

1. Saint CP, Ho L. A PCR test for identification and discrimination of *Legionella longbeachae* serogroups 1 and 2. J Microbiol Metod. 1999; 37: 245–3.
2. Huang L, Boyd D, Amyot WM, Hempstead AD, Luo Z, O'Connor TJ, Chen C, Machner M, Montminy T, Isberg RR. The E Block motif is associated with *Legionella pneumophila* translocated substrates. Cell Microbiol. 2011; 13: 227–45.
3. Papciak D, Zamorska J. Podstawy biologii i biotechnologii środowiskowej. Oficyna Wydawnicza Politechniki Rzeszowskiej, Rzeszow, 2005.p.54–69.
4. Heuner K, Steinert M. The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype. Int J Med Microbiol. 2003; 293: 133–43.
5. Yañez M, Carrasco–Serrano AC, Barbera V, Catalan V. Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real – time PCR amplification of the dotA gene. Appl Environ Microbiol. 2005; 71: 3433–41.
6. Declerck P. Biofilms: the environmental playground of *Legionella pneumophila*. Environ Microbiol. 2010; 12: 557–66.
7. Steinert M, Hentschel U, Hacker J. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol Rev. 2002; 26: 149–62.
8. Flemming HC, Wingeneder J, Mayer C, Körstgens V, Borchard W. Cohesiveness in biofilm matrix polymers. In: Allison DG, Lapin – Scott HM, Wilson M. Society for General Microbiology Symposium: Community Structure and Co – Operation in Biofilms. University Press, Cambridge, UK: 2000.p.88–106.
9. Grimm D, Ludwig W, Brandt BC, Michel R, Schleifer KH, Hacker J, Steinert M. Development of 18S rRNA-targeted Oligonucleotide Probes for Specific Detection of *Hartmannella* and *Naegleria* in *Legionella* – positive Environmental Samples, Syst. Appl. Microbiol. 2001; 24: 76–82.
10. Barbaree JM, Sanchez A, Sanden GN. Tolerance of *Legionella* Species to Sodium Chloride. Curr Microbiol. 1983; 9: 1–5.
11. Palmer CJ, Tsai Y, Paszko-Kolva C, Mayer C, Sangermano LR. Detection of *Legionella* Species in Sewage and Ocean Water by Polymerase Chain Reaction, Direct Fluorescent-Antibody, and Plate Culture Methods. Appl Environ Microbiol. 1993; 93: 3618–24.
12. Guyard C, Low DE. *Legionella* infections and travel associated legionellosis. Travel Med Infect Dis. 2011; 9: 176–88.
13. Misiurkiewicz-Stypułkowska H, Krogulska B, Pancer K, Matuszewska R. Metodyka wykrywania i oznaczania bakterii z rodzaju *Legionella* w środowisku wodnym i w materiale klinicznym. Wyd. Met.PZH, Warszawa 2001.p.33–47.
14. Declerck P, Behets J, Hoef V, Ollevier F. Detection of *Legionella* spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. Water Res. 2007; 41: 3159–67.
15. Huang SW, Hsu BM, Wu SF, Fan CW, Shih FC, Lin YC, Ji DD. Water quality parameters associated with prevalence of *Legionella* in hot spring facility water bodies. Water Res. 2010; 44: 4805–11.
16. Exner M, Hartemann P. Summary of the second meeting of the International Forum on Water Hygiene in Buildings (IFOWAHB) from 01 to 02.06.2007 in Stockholm, Meeteing Report. Int J Hyg Environ Health 2009; 212: 449–458.
17. Meenhorst PL, Reingold AL, Groothuis DG, Gorman GW, Wilkinson HW, McKinney RM, Feeley JC, Brenner DJ, Van Furth R. Water – related nosocomial pneumonia caused by *Legionella pneumophila* serogroups 1 and 10. J Infect Dis. 1985; 152: 356–64.
18. Patterson WJ, Seal DV, Curran E, Sinclair TM, McLuckie JC. Fatal nosocomial Legionnaires disease: relevance of contamination of hospital water supply by temperature – dependent buoyancy – driven flow from spur pipes. Epidemiol Infec. 1994; 112: 513–25.