

# Efficacy of the detection of *Legionella* in hot and cold water samples by culture and PCR. I. Standardization of methods

Angelina Wójcik-Fatla<sup>1</sup>, Nimfa Maria Stojek<sup>2</sup>, Jacek Dutkiewicz<sup>1</sup>

<sup>1</sup> Department of Zoonoses, Institute of Rural Health, Lublin, Poland

<sup>2</sup> Department of Water and Soil Safety, Institute of Rural Health, Lublin, Poland

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## Abstract

The aim of the present study was: – to compare methods for concentration and isolation of *Legionella* DNA from water; – to examine the efficacy of various modifications of PCR test (PCR, semi-nested PCR, and real-time PCR) for the detection of known numbers of *Legionella pneumophila* in water samples artificially contaminated with the strain of this bacterium and in randomly selected samples of environmental water, in parallel with examination by culture. It was found that filtration is much more effective than centrifugation for the concentration of DNA in water samples, and that the Qiamp DNA Mini-Kit is the most efficient for isolation of *Legionella* DNA from water. The semi-nested PCR and real-time PCR proved to be the most sensitive methods for detection of *Legionella* DNA in water samples. Both PCR modifications showed a high correlation with recovery of *Legionella* by culture ( $p < 0.01$ ), while no correlation occurred between the results of one-stage PCR and culture ( $p > 0.1$ ).

## Key words

*Legionella*, detection, methods, water, culture, PCR, efficacy

## INTRODUCTION

Bacteria belonging to the genus *Legionella* occur commonly in water and after inhalation of water aerosol may cause in humans severe pneumonia or flu-like Pontiac fever [1]. The presence and concentration of these bacteria in water samples is identified mostly by culture on specific media. However, this specific method is time-consuming and does not detect viable but non-cultivable bacteria in a water sample [2]. Hence, a number of sensitive PCR-based methods for detection of these bacteria in water has been developed, including PCR, semi-nested PCR, nested-PCR and real-time PCR, that allows for quantitative determination of the concentration of *Legionella* in water [2, 3, 4, 5, 6, 7, 8, 9, 10]. The recent modifications of the PCR test allow discrimination between live and dead bacteria [11, 12].

The aim of the present study was to examine the efficacy of various modifications of the PCR test (PCR, semi-nested PCR, and real-time PCR) for the detection of known numbers of *Legionella pneumophila* in water samples artificially contaminated with the strain of this bacterium, and in randomly selected samples of environmental water, in parallel with examination by culture. Our final goal was to choose the optimal PCR modification for determining the presence and concentration of *Legionella* in samples of potable water collected in eastern Poland.

## MATERIALS AND METHODS

**Efficacy of the concentration of water samples by filtration and centrifugation.** The samples of sterile water (MiliQ) were spiked with one colony of *Legionella pneumophila* (serogroup 2-14) and serial dilutions were made from  $10^{-1}$ - $10^{-7}$ . One set of dilutions was concentrated by filtration through cellulose filters (Millipore Corporation, Billerica, MA, USA) of pore size 0.45  $\mu\text{m}$ . The sediment was scraped from the filter, suspended in 3-4 ml PBS, transferred to sterile Eppendorf tubes, and centrifuged at  $5,000 \times g$  for 10 min. Supernatant was removed and sediment was suspended in 20-30  $\mu\text{l}$  PBS for further processing.

The second set of dilutions was concentrated only by centrifugation, first at  $2,700 \times g$  for 30 min and then at  $20,000 \times g$  for 10 min. Supernatant was removed and sediment used for further processing.

From the sediments obtained by filtration and centrifugation, DNA was isolated with the use of Qiamp DNA Mini-Kit, USA. The quality of isolated DNA was measured by determination of absorbancy at the wave lengths  $A_{260}$  nm and  $A_{280}$  nm (ratio of these values between 1.7 and 1.9 indicates a high quality of the product) and by measuring DNA in ng/ $\mu\text{l}$  by a spectrophotometer NanoDrop ND-1000 (Syngen). The efficacy of DNA isolations by filtration and centrifugation was also assessed by 3 modifications of PCR (Tab. 1).

**Efficacy of various kits for isolation of DNA from water samples.** The concentrated by filtration native samples of tap and well water of the volume of 50-100  $\mu\text{l}$  were incubated with lysing buffer, after which DNA was isolated by 3 commercial kits: Qiamp DNA Mini Kit (Qiagen, USA), NucleoSpin (Macherey-Nagel, France), and NucliSens Magnetic Extraction Reagents (Biomérieux, France). The efficacy of

Address for correspondence: Angelina Wójcik-Fatla, Department of Zoonoses, Institute of Rural Health, Jaczewskiego 2, 20-090 Lublin, Poland.  
E-mail: afatla@poczta.onet.pl

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**Table 1.** DNA isolation from water samples artificially spiked with standard strain of *Legionella pneumophila* (sg. 2-14) using centrifugation and filtration techniques

| No.  | Dilution                       | DNA          |                       | Semi-nested PCR ( <i>dotA</i> ) |       | PCR (16S rRNA) | Real-time PCR |
|--|--------------------------------|--------------|-----------------------|---------------------------------|-------|----------------|---------------|
|  |                                | A260m/A280nm | Concentration (ng/μl) | PCR 1                           | PCR 2 |                |               |
| <b>Centrifugation</b>  |                                |              |                       |                                 |       |                |               |
| Serial dilution of water samples spiked with one colony of <i>Legionella pneumophila</i> |                                |              |                       |                                 |       |                |               |
| 1  | 1 × 10 <sup>-1</sup>           | 1.52         | 2.03                  | +                               | +     | +              | +             |
| 2  | 1 × 10 <sup>-2</sup>           | 1.63         | 1.60                  | +                               | +     | +              | +             |
| 3  | 1 × 10 <sup>-3</sup>           | 1.31         | 1.45                  | +                               | +     | +              | +             |
| 4  | 1 × 10 <sup>-4</sup>           | 1.82         | 1.51                  | (-)                             | +     | +              | +             |
| 5  | 1 × 10 <sup>-5</sup>           | 1.42         | 1.21                  | (-)                             | (-)   | (-)            | (-)           |
| 6  | 1 × 10 <sup>-6</sup>           | 1.08         | 0.4                   | (-)                             | (-)   | (-)            | (-)           |
| 7  | 1 × 10 <sup>-7</sup>           | 1.04         | 0.56                  | (-)                             | (-)   | (-)            | (-)           |
| 8  | Negative control: MilliQ water |              |                       | (-)                             | (-)   | (-)            | (-)           |
| <b>Filtration</b>  |                                |              |                       |                                 |       |                |               |
| Serial dilution of water samples spiked with one colony of <i>Legionella pneumophila</i> |                                |              |                       |                                 |       |                |               |
| 1  | 1 × 10 <sup>-1</sup>           | 1.95         | 2.66                  | +                               | +     | +              | +             |
| 2  | 1 × 10 <sup>-2</sup>           | 1.85         | 2.54                  | +                               | +     | +              | +             |
| 3  | 1 × 10 <sup>-3</sup>           | 1.76         | 1.98                  | +                               | +     | +              | +             |
| 4  | 1 × 10 <sup>-4</sup>           | 1.4          | 1.84                  | +                               | +     | +              | +             |
| 5  | 1 × 10 <sup>-5</sup>           | 1.3          | 1.95                  | +                               | +     | +              | +             |
| 6  | 1 × 10 <sup>-6</sup>           | 0.96         | 1.53                  | (-)                             | +     | +              | +             |
| 7  | 1 × 10 <sup>-7</sup>           | 0.84         | 1.43                  | (-)                             | +     | (-)            | +             |
| 8  | Negative control: MilliQ water |              |                       | (-)                             | (-)   | (-)            | (-)           |

+ = positive result; (-) = negative result.

**Table 2.** Comparison of kits for DNA isolation on the basis of PCR results

| No. | Examined sample   | Qiamp DNA Mini Kit  |                | NucleoSpin          |                | NucliSens           |                |
|-----|-------------------|---------------------|----------------|---------------------|----------------|---------------------|----------------|
|     |                   | PCR ( <i>dotA</i> ) | PCR (16S rRNA) | PCR ( <i>dotA</i> ) | PCR (16S rRNA) | PCR ( <i>dotA</i> ) | PCR (16S rRNA) |
| 1.  | Well water        | (-)                 | (-)            | (-)                 | (-)            | (-)                 | (-)            |
| 2.  | Well water        | +                   | +              | +                   | +              | +                   | +              |
| 3.  | Well water        | +                   | +              | +                   | (-)            | (-)                 | +              |
| 4.  | Well water        | +                   | +              | (-)                 | +              | (-)                 | (-)            |
| 5.  | Well water (deep) | +                   | +              | (-)                 | (-)            | (-)                 | (-)            |
| 6.  | Well water (deep) | +                   | +              | (-)                 | (-)            | (-)                 | (-)            |
| 7.  | Tap water (hot)   | -                   | +              | (-)                 | (-)            | (-)                 | (-)            |
| 8.  | Tap water (hot)   | +                   | +              | (-)                 | (-)            | +                   | +              |
| 9.  | Tap water (hot)   | (-)                 | (-)            | (-)                 | (-)            | (-)                 | (-)            |
| 10. | Tap water (hot)   | +                   | +              | (-)                 | (-)            | (-)                 | (-)            |

+ = positive result; (-) = negative result.

DNA isolation by individual kits was assessed by PCR (*dotA*) and PCR (16SrRNA) (Tab. 2).

**Isolation and identification of *Legionella* from water samples by culture.** From each water sample, volumes of 100 ml each were filtered through Millipore cellulose filters of pore size 0.45 μm for recovery of *Legionella* by culture and by PCR. Filters assigned for culture were washed for 5 min in acid buffer (pH 2.2), then rinsed in Ringer solution (Merck, Darmstadt, Germany), and finally placed on the isolation agar medium. The buffered charcoal yeast extract (BCYE) agar medium supplemented with Growth Supplement SR 110 A and Selective GVPC Supplement SR 152 E (Oxoid, Basingstoke, Hampshire, UK) [13, 14, 15, 16] was used for

isolation of *Legionella* (further referred to as GVPC medium). Inoculated agar plates were incubated for 7 days at 37°C with an every day check of growth. Colonies of Gram-negative bacteria grown after 4-7 days were isolated and examined for ability to grow on media with and without cysteine.

Strains unable to grow on media without cysteine were considered as suspected *Legionella* strains. The isolates were determined to the species and serogroup level with the use of the Legionella Latex Test Kit (Oxoid, Basingstoke, Hampshire, UK) which enables, on the basis of microagglutination with latex particles sensitised with specific rabbit antibodies, a separate identification of *Legionella pneumophila* serogroup 1, *Legionella pneumophila* serogroups 2-14, and *Legionella* spp. (a complex group including: *L. longbeache* serogroups 1 and 2, *L. bozemanii* serogroups 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei* and *L. anisa*) [13]. Only isolates producing a positive reaction in the latex test were considered as strains of *Legionella*.

**One-stage PCR.** For this, and for other modifications of PCR, DNA was isolated from the sediment on Millipore filters using Qiamp DNA Mini-Kit (Qiagen, USA). The test was carried out by amplification of gene fragment 16SrRNA with the use of primers pair JFP and JRP. The sequences of the used primers were as follows:

JFP: 5' -AGGGTTGATAGGTTAAGAGC-3'  
JRP: 5' -CCAACAGCTAGTTGACATCG-3'

The reaction mix of the total volume 50 μl contained: 5 μl reaction buffer containing 15 mM MgCl<sub>2</sub>, 3 mM of additional MgCl<sub>2</sub> (Qiagen), 200 μM each dATP, dGTP, dCTP, and dTTP, 1 μM of primers JFP and FRP, 1 U UDG (Uracil-DNA-Glycosylase), 2.8 U *Taq* DNA polymerase (Qiagen, USA), 5 μl matrix DNA and nuclease-free water (Applied Biosystems).

Amplification was carried out in a C1000 Thermal Cycler (BioRad, USA) and comprised: • initial incubation (37°C, 10 min, action of uracil glycosylase); • initial denaturation (95°C, 20 min); • 38 cycles, each comprising of: denaturation (94°C, 45 sec), annealing (57°C, 45 sec), elongation (72°C, 45 sec); • final elongation (72°C, 1 hr).

The products of amplification were identified in 2% agarose gel after electrophoresis in standard conditions and staining with ethidium bromide solution (2 μg/ml). The size of the amplified DNA fragments was 386 bp.

**Semi-nested PCR.** The semi-nested PCR was carried out by amplification of gene fragment *dotA* with the use of primers pair dotF and dotRM (PCR 1) and reamplification of the PCR 1 product with the use of primers pair dotRM and dotFK (PCR 2, semi-nested).

The sequences of the used primers were as follows:

dotF: 5' -ATTGTCTCGCGGATTGC-3'  
dotRM: 5' -CTTCCATTGAGTTTCACCAAATCA-3'  
dotFK: 5' -GGTGATGGTTAATAATGATCCGGC-3'

The test was carried out according to Yanez *et al.* [17] in own modification. The modification relied on the change of reaction mix which increased the sensitivity of the test. The reaction mix of the total volume 25 μl contained: 2.5 μl reaction buffer containing 15 mM MgCl<sub>2</sub>, 1.5 μl of additional MgCl<sub>2</sub> in the concentration of 25 mM (Qiagen),

1.25 µl of 10 µM primers dotF and dotRM, 2.5 µl dNTP in the concentration of 2 mM (Fermentas, Vilnius, Lithuania), 0.5 U *Taq* DNA polymerase (Qiagen, USA), 2 µl matrix DNA and nuclease-free water (Applied Biosystems).

Amplification was carried out in the C1000 Thermal Cycler (BioRad, USA) and comprised: • initial denaturation (94°C, 4 min); • 35 cycles, each comprising of: denaturation (94°C, 30 sec), annealing (58°C, 30 sec), elongation (72°C, 1 min); • final elongation (72°C, 10 min).

In the reamplification (semi-nested PCR, PCR II) the primers dotRM and dot FK were used in the same concentration as during the first reaction. The conditions of this reaction were the same as in first amplification, except that the temperature of annealing was 62°C.

The products of amplification and reamplification were identified in 2% agarose gel after electrophoresis in standard conditions and staining with ethidium bromide solution (2 µg/ml).

The sizes of the amplified DNA fragments after amplification and reamplification were 440 bp and 387 bp, respectively.

**Real-time PCR.** The test was carried out with the use of commercial set *Legionella* spp. Quantitative Detection Kit (ielab kits, Spain, distributed by Applied Biosystems) with the use of StepOne device (Applied Biosystems, USA). The commercial mix contained primers specific for *Legionella* spp. TaqMan Universal Master Mix, fluorescent probes, IPC (Internal Positive Control – plasmid DNA). To 15 µl of reaction mix was added: • 10 µl of matrix DNA; • 10 µl of nuclease-free water (negative control); • 10 µl of positive control: 6 successive dilutions of positive control (strain of *Legionella pneumophila*) at the initial concentration of  $1 \times 10^6$  genome units/µl.

All samples were tested by 3-fold repetitions. Results were read with the use of standard slope, provided by the producer (slope points were: 100,000, 10,000, 1,000, 100, 10, 1). Amplification comprised: • 50°C, 2 min; • 95°C, 10 min; • 42 cycles, each comprising of: 95°C, 15 sec, and 60°C, 1 min.

**Comparison of *Legionella* detection by culture and PCR.** In the first stage, the samples of sterile water (MiliQ) of the volume of 10 ml were spiked with one colony of *Legionella pneumophila* (serogroup 2-14) and serial dilutions from  $10^{-1}$  to  $10^{-7}$  were made in 2 repetitions. From each dilution, inoculation of cultures and isolation of DNA for performance of 3 PCR-modifications (PCR, semi-nested PCR and real-time PCR) were made (Tab. 3).

In the second stage, 40 native samples of potable water (35 samples of hot tap water from an urban municipal water supply system and 5 samples of cold well water) were tested

for the presence and concentration of *Legionella* by culture and by 3 modifications of PCR: one stage PCR (16rRNA), semi-nested PCR (*dotA*), and real-time PCR (Tab. 4).

**Statistical analysis.** Data were analysed by the Shapiro-Wilk W-test for distribution, Spearman's rank order test for correlation, Wilcoxon matched pairs test, and Student's t-test with the use of STATISTICA for Windows v. 5.0 package (StatSoft Inc., Tulsa, Oklahoma, USA). The value  $p < 0.05$  was considered significant.

## RESULTS

**Efficacy of filtration and centrifugation in the isolation of *Legionella* DNA.** Filtration proved to be a much more efficient method than centrifugation (Tab. 1). The concentration of DNA (ng/µl) was significantly ( $p=0.018$ ) greater in the samples separated by filtration compared to those separated by centrifugation. This was also confirmed by the PCR results. In the samples separated by centrifugation, the *Legionella* DNA was detected only down to the dilution  $10^{-4}$  by all 3 PCR-modifications (one stage PCR, semi-nested PCR, real-time PCR), whereas in the samples separated by filtration it was detected down to the dilution  $10^{-7}$  by semi-nested and real-time PCR, and down to the dilution  $10^{-6}$  by one-stage PCR (Tab. 1).

**Efficacy of various kits in the isolation of *Legionella* DNA from water samples.** The PCR-results presented in Table 2 clearly indicate that the kit Qiamp DNA Mini-Kit appeared to be much more efficient than the NucleoSpin and NucliSens kits for isolation of *Legionella* DNA, both from cold well water and hot tap water.

**Comparison of the culture vs. PCR results in water samples artificially spiked with *Legionella pneumophila*.** The results of *Legionella* detection by culture complied with those obtained by PCR (Tab. 3). Presence of *Legionella* was detected by culture and one-stage PCR down to the dilution  $10^{-6}$ . The semi-nested PCR and real-time PCR proved to be more sensitive, down to the dilution  $10^{-7}$ . A significant correlation was found between the results of culture and real-time PCR ( $p=0.000533$ ).

**Comparison of the culture vs. PCR results in the native samples of tap water (hot) and well water (cold).** The results of *Legionella* detection by culture and by 3 modifications of PCR in 40 samples of hot and cold water are presented in Table 3. A highly significant correlation was found between

**Table 3.** Examination of water samples artificially spiked with *Legionella pneumophila* strain (sg. 2-14) with the use of culture and PCR.

| No. | Sample, dilution   | Culture (colony forming units/100 ml) |                | PCR (16S rRNA) | PCR ( <i>dotA</i> ) |            | Real-time PCR (number of genome units/µl) |                |
|-----|--------------------|---------------------------------------|----------------|----------------|---------------------|------------|---|----------------|
|     |                    | 1st repetition                        | 2nd repetition |                | PCR                 | Nested-PCR | 1st repetition                            | 2nd repetition |
| 1   | 1 colony           | +                                     | +              | +              | +                   | +          | >1,000,000                                | >1,000,000     |
| 2   | $1 \times 10^{-1}$ | +                                     | +              | +              | +                   | +          | 769,370.2                                 | 748,905.3      |
| 3   | $1 \times 10^{-2}$ | +                                     | +              | +              | +                   | +          | 53,733.4                                  | 56,951.9       |
| 4   | $1 \times 10^{-3}$ | +                                     | +              | +              | +                   | +          | 7,461.5                                   | 7,534.8        |
| 5   | $1 \times 10^{-4}$ | +                                     | +              | +              | +                   | +          | 2,021.6                                   | 2,210.9        |
| 6   | $1 \times 10^{-5}$ | +                                     | +              | +              | +                   | +          | 371.3                                     | 371.1          |
| 7   | $1 \times 10^{-6}$ | +                                     | (-)            | +              | (-)                 | +          | 108.5                                     | 101.5          |
| 8   | $1 \times 10^{-7}$ | (-)                                   | (-)            | (-)            | (-)                 | +          | 10.2                                      | 10.7           |

+ = positive result; (-) = negative result; (nq.) = not quantified (overgrowth); (ngr.) = no growth.

**Table 4.** Results of the examination of native samples of tap water and well water for the presence of *Legionella pneumophila* and *Legionella* spp., using culture and PCR methods

| No. | Water sample    | Culture: species and serogroup of <i>Legionella</i> (CFU/100 ml) | PCR (16S rRNA) | PCR ( <i>dotA</i> ) |            | Real-time PCR (genome units, GU/ $\mu$ l) |
|-----|-----------------|--|----------------|---------------------|------------|---|
|     |                 |  |                | PCR                 | Nested-PCR |   |
| 1   | Tap water (hot) | + <i>L. pneum.</i> 2-14 (30)                                     | (-)            | (-)                 | +          | + (358.9)                                 |
| 2   | Tap water (hot) | (-)  | (-)            | (-)                 | (-)        | (-)                                       |
| 3   | Tap water (hot) | + <i>L. pneum.</i> 2-14 (10)                                     | (-)            | +                   | +          | (-)                                       |
| 4   | Tap water (hot) | + <i>L. pneum.</i> 2-14 (10)                                     | (-)            | (-)                 | (-)        | (-)                                       |
| 5   | Tap water (hot) | (-)  | +              | (-)                 | +          | + (5.92)                                  |
| 6   | Well water      | (-)  | +              | (-)                 | +          | + (10.85)                                 |
| 7   | Well water      | (-)  | (-)            | (-)                 | (-)        | (-)                                       |
| 8   | Well water      | (-)  | +              | (-)                 | (-)        | (-)                                       |
| 9   | Well water      | (-)  | (-)            | (-)                 | (-)        | (-)                                       |
| 10  | Well water      | (-)  | +              | (-)                 | (-)        | (-)                                       |
| 11  | Tap water (hot) | (-)  | (-)            | (-)                 | (-)        | + (4.3)                                   |
| 12  | Tap water (hot) | + <i>L. pneum.</i> 1, 2-14<br><i>Leg. spp.</i> (>200)            | +              | +                   | +          | + (>50,000)                               |
| 13  | Tap water (hot) | (-)  | +              | (-)                 | +          | + (4.7)                                   |
| 14  | Tap water (hot) | + <i>L. pneum.</i> 1, 2-14<br><i>Leg. spp.</i> (>100)            | (-)            | (-)                 | +          | + (>100,000)                              |
| 15  | Tap water (hot) | (-)  | +              | +                   | +          | + (7.69)                                  |
| 16  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (> 100)                                  | +              | (-)                 | +          | + (>100,000)                              |
| 17  | Tap water (hot) | (-)  | +              | (-)                 | +          | + (0.64)                                  |
| 18  | Tap water (hot) | + <i>L. pneum.</i> 1, 2-14<br><i>Leg. spp.</i> (>100)            | +              | (-)                 | +          | + (>100,000).                             |
| 19  | Tap water (hot) | (-)  | (-)            | (-)                 | (-)        | (-)                                       |
| 20  | Tap water (hot) | (-)  | (-)            | +                   | +          | + (40,000)                                |
| 21  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (>100)                                   | +              | +                   | +          | + (40,000)                                |
| 22  | Tap water (hot) | + <i>L. pneum.</i> 2-14<br><i>Leg. spp.</i> (> 100)              | (-)            | +                   | +          | + (95,000)                                |
| 23  | Tap water (hot) | (-)  | +              | +                   | +          | + (1.34)                                  |
| 24  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (>100)                                   | +              | +                   | +          | + (>100,000)                              |
| 25  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (>100)                                   | +              | +                   | +          | + (2.8)                                   |
| 26  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (50)                                     | +              | (-)                 | +          | + (12,000)                                |
| 27  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (>200)                                   | +              | (-)                 | +          | + (0.59)                                  |
| 28  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (100)                                    | +              | +                   | +          | + (1.77)                                  |
| 29  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (>200)                                   | +              | +/-                 | +          | + (>100,000)                              |
| 30  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (>100)                                   | +              | +/-                 | +          | + (0.91)                                  |
| 31  | Tap water (hot) | + <i>L. pneum.</i> 1, 2-14<br><i>Leg. spp.</i> (>100)            | +              | +/-                 | +          | + (0.76)                                  |
| 32  | Tap water (hot) | + <i>L. pneum.</i> 1, 2-14<br><i>Leg. spp.</i> (100)             | (-)            | (-)                 | +          | + (41,000)                                |
| 33  | Tap water (hot) | (-)  | +              | (-)                 | +          | + (0.73)                                  |
| 34  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (50)                                     | +              | (-)                 | (-)        | (-)                                       |
| 35  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (50)                                     | +              | +/-                 | +          | + (480)                                   |
| 36  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (4)                                      | +              | +                   | +          | + (4.76)                                  |
| 37  | Tap water (hot) | + <i>L. pneum.</i> 2-14 (80)                                     | +              | +/-                 | +          | + (1.18)                                  |
| 38  | Tap water (hot) | (-)  | +              | (-)                 | (-)        | + (1.60)                                  |
| 39  | Tap water (hot) | (-)  | (-)            | (-)                 | +          | (-)                                       |
| 40  | Tap water (hot) | (-)  | (-)            | (-)                 | (-)        | (-)                                       |

CFU = colony forming units; + = positive result; +/- = borderline positive result; (-) = negative result.

the results of culture on one side and of semi-nested PCR and real-time PCR on the second side ( $p=0.00127$ , and  $p=0.00035$ , respectively). No significant correlation appeared between the results of culture and of one-stage PCR ( $p=0.11881$ ). The levels of *Legionella* in the cold well water were distinctly lower compared to hot tap water.

## DISCUSSION

The results of the study show a significant correlation between the detection of *Legionella* in water samples by culture and by the advanced modifications of PCR: semi-nested PCR and real-time PCR, thus confirming the results of earlier authors [2, 3, 5, 6, 7, 10]. The real-time PCR creates the best possibilities for quantitation of the results, it is, however, expensive and prone to inhibition by water impurities.

In conclusion, based on the results of the presented study, we selected the following methodological conditions for further investigation of *Legionella* occurrence in water by PCR:

- concentration of DNA by filtration;
- use of Qiamp DNA Mini-Kit for isolation of *Legionella* DNA from water;
- use of the semi-nested PCR for detection of *Legionella*.

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