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Efficacy of the detection of *Legionella* in hot and cold water samples by culture and PCR. II. Examination of native samples from various sources

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Abstract

A total of 123 water samples were examined in parallel by culture and semi-nested PCR for the presence of *Legionella*. They comprised: 35 samples of hot water distributed by the urban municipal water supply system (MWSS) taken in institutions, 45 samples of hot water distributed by urban MWSS taken in dwellings, 27 samples of cold water distributed by rural MWSS taken in dwellings, and 16 samples of cold well water taken in rural areas. The greatest frequency of the isolation of *Legionella* by culture (88.6%) was recorded in the samples of hot water from the urban institutions, having been greater compared to all other sources ($p<0.001$). The frequency of *Legionella* isolation from hot water in urban dwellings (28.9%) was significantly greater compared to the combined value (2.3%) for cold water from rural MWSS and wells ($p<0.001$). Strains belonging to *Legionella pneumophila* serogroups 2-14 predominated in the examined samples, while strains of *L. pneumophila* serogroup 1 and strains of *Legionella* spp. (other than *L. pneumophila*) were 3-fold less numerous. The rates of positive findings in the semi-nested PCR (stage 2) were greater than culture isolations in all kinds of samples, except for urban institutions. The correlation between the culture and PCR results was positive for samples of hot water from urban MWSS ($p<0.01$), but not for samples of cold water from rural MWSS and wells ($p>0.5$). A significant correlation was found between rates of PCR-positive results and numbers of *Legionella pneumophila* serogroups 2-14 strains, but not for other *Legionella* serogroups or species. In conclusion, our results support the opinion that though PCR cannot be a substitute for the isolation of *Legionella* by culture, it could be regarded as an useful complementary method.

Key words

Legionella, detection, water, culture, PCR, efficacy, water supply systems, well water

INTRODUCTION

The genus *Legionella* comprises fastidious Gram-negative bacteria widely distributed in various aquatic and soil biotopes, including water supply systems [1]. The bacteria proliferate inside fresh water amoebae and biofilms. They may cause respiratory diseases in humans, mostly after exposure to the inhalation of bacteria-laden droplet aerosol, less often by the oral route through drinking water, and through traumatized skin or mucous membranes [2]. As the disease risk depends on the concentration of *Legionella* in potable water, the threshold limit values have been established worldwide, in Poland at the level of 100 cells per 100 ml water [3].

The isolation of *Legionella* by culture is time-consuming and does not warrant the detection of this bacterium in a water sample. Thus, a number of sensitive molecular methods for detection of these bacteria in water has been developed, including PCR, semi-nested PCR, nested-PCR and real-time PCR that allow for quantitative determination of the concentration of *Legionella* in water [4, 5, 6, 7, 8, 9, 10].

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In the first part of this study [11], we examined the efficacy of various modifications of PCR test for the detection of known numbers of *Legionella* in the water samples artificially contaminated with the reference strains of this bacterium. Based on the obtained results, we chose the semi-nested PCR test as a reliable tool for examination of the native samples of water from various sources, in parallel with examination by culture. The aim of the present study was statistical comparison of the results.

MATERIALS AND METHODS

Samples of water. A total of 123 samples of water, used as a potable water for consumption, were taken during the summer months (June – August) in 2010-2011 from various places located in the Lublin province of eastern Poland. Water samples were taken into sterile glass bottles of the volume of 700 ml at the following sites:

- 6 samples of cold well water were taken directly (with use of a pail) from 6 private household wells located on farms in one village.
- 10 samples of cold well water were collected from taps of the 10 private water supply systems (PWSS) conducting untreated and unheated water from household wells to outlets within farm buildings located in one village.

- 27 samples were taken on 20 farms in 3 villages from cold-water taps of the municipal water supply system (MWSS) distributing treated (chlorinated) groundwater. The taps were equipped with aerators or other endings for better outflow of water.
- 45 samples were collected in 45 dwellings located in the city of Lublin, from hot-water taps of the MWSS distributing treated (chlorinated) groundwater, pumped from the depth of 40-100 m. The taps were equipped with aerators or other endings for better outflow of water.
- 35 samples were collected in 2 institutions located in the city of Lublin, from hot-water taps of the MWSS distributing treated (chlorinated) groundwater, pumped from the depth of 40-100 m. The taps were equipped with aerators or other endings for better outflow of water.

Processing of samples. From each water sample, 2 volumes of 100 ml each were filtered through cellulose filters (pores 0.45 μm, Millipore Corporation, Billerica, MA, USA) for recovery of *Legionella* by culture and by semi-nested 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 isolation agar medium.

Isolation and identification of *Legionella* strains. Methodology was described in part I of this study [11]. Briefly, the buffered charcoal yeast extract (BCYE) agar medium supplemented with the Growth Supplement SR 110 A and the Selective GVPC Supplement SR 152 E (Oxoid, Basingstoke, Hampshire, UK) [12] was used for the isolation of *Legionella*. The isolates were determined to the species and serogroup level with the use of the Legionella Latex Test Kit (Oxoid, Basingstoke, Hampshire, UK) which enabled a separate identification of *Legionella pneumophila* serogroup 1, *Legionella pneumophila* serogroups 2-14, and *Legionella* spp.

Semi-nested PCR. Methodology was based on part I of this study [11]. Briefly, DNA was isolated from the sediment on Millipore filters using Qiamp DNA Mini Kit (Qiagen, USA). The semi-nested PCR was carried out by amplification of gene fragment *dot*A 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 test was carried out according to Yanez *et al.* [13] in own modification. The sizes of the amplified DNA fragments after amplification and reamplification were 440 bp and 387 bp, respectively.

Statistical analysis. The data were analysed by Shapiro-Wilk W-test for distribution, χ^2 test, 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

Frequency of the isolation of *Legionella* by culture. The greatest frequency of the isolation of *Legionella* by culture (88.6%) was recorded in the samples of hot water from the urban institutions, distributed by the municipal water supply system (MWSS) (Tab. 1). This was significantly greater compared to all other sources ($p<0.001$), including hot water from urban dwellings distributed by MWSS, cold water from rural dwellings distributed by MWSS, and cold water taken from wells in rural areas. The frequency of *Legionella* isolation from hot water in urban dwellings (28.9%) was significantly greater compared to the combined value (2.3%) for cold water from rural MWSS and wells ($p<0.001$).

Species composition of *Legionella* isolates. Strains belonging to *Legionella pneumophila* serogroups 2-14 clearly dominated and were isolated from all water samples positive for *Legionella*. Strains of *Legionella pneumophila* serogroup 1 and *Legionella* spp. (comprised of species other than *L. pneumophila*) were isolated only from samples of warm water in the range 13.3-28.6%, always in the association with *L. pneumophila* serogroups 2-14 (Tab. 1).

Detection of *Legionella* by semi-nested PCR. At reaction stage 1, the rates of positive findings obtained by PCR were lowered than culture in all water sources. By contrast, at reaction stage 2, the incidence of PCR-positive results was greater than culture in all water sources except urban MWSS institutions (Tab. 1). The difference proved to be borderline, insignificant for urban MWSS dwellings ($p=0.055$) and total samples ($p=0.05850$), and significant for rural well water ($p=0.002$).

Relationship between results obtained by culture and semi-nested PCR. A highly significant correlation between culture and the second stage of semi-nested PCR was found for samples of hot water from institutions ($p<0.0001$) and dwellings ($p<0.01$), as well as for the total water samples investigated ($p<0.00001$) (Tab. 2). The correlation between culture and the first stage of PCR was less distinct, although

Table 1. Occurrence of *Legionella* in water samples from various sources determined by culture and semi-nested PCR

Source of water	Culture: frequency, species composition				Semi-nested PCR	
	Positive (%)	*Legionella p.* Sg. 2-14	*Legionella p.* Sg. 1*	*Legionella* spp.*	Reaction 1 Positive (%)	Reaction 2 Positive (%)
Urban MWSS, institutions, hot water N=35	31 (88.6%)	31 (88.6%)	7 (20.0%)	10 (28.6%)	14 (40.0%)	26 (74.3%)
Urban MWSS, dwellings, hot water N=45	13 (28.9%)	13 (28.9%)	9 (20.0%)	6 (13.3%)	7 (15.6%)	22 (48.9%)
Rural MWSS dwellings, cold water N=27	0	0	0	0	0	2 (7.4%)
Wells in rural areas, dwellings, cold water N=16	1 (6.3%)	1 (6.3%)	0	0	1 (6.3%)	10 (62.5%)
Total samples from urban and rural areas N=123	45 (36.6%)	14 (36.6%)	16 (13.0%)	16 (13.0%)	22 (17.9%)	60 (48.8%)

N=Numbers of examined samples;
*isolated only together with *Legionella pneumophila* sg. 2-14 strains.

Table 2. Conformity of detection of *Legionella* in water samples by culture and semi-nested PCR

Source of water			Type of PCR reaction			
Urban MWSS institutions hot water N = 35			PCR 1 pos.	PCR 1 neg.	PCR 2 pos.	PCR 2 neg.
	Culture	Positive	13	18	24	7
		Negative	1	3	2	2
		Conformity	45.7%		74.3%	
		Significance	χ^2 = 0.01, p=0.914, (-)		χ^2 = 17.75, p<0.0001, +++	
Urban MWSS dwellings hot water N = 45						
	Culture	Positive	5	8	11	2
		Negative	2	30	11	21
		Conformity	77.8%		71.1%	
		Significance	χ^2 = 5.06, p=0.025, +		χ^2 = 7.44, p=0.006 ++	
Rural MWSS dwellings cold water N = 27						
	Culture	Positive	0	3	0	3
		Negative	0	24	2	22
		Conformity	88.9%		81.5%	
		Significance	Could not be computed		χ^2 = 0.42, p=0.516, (-)	
Well water cold water N = 16						
	Culture	Positive	0	1	1	0
		Negative	0	15	8	7
		Conformity	93.7%		50.0%	
		Significance	Could not be computed		χ^2 = 0.025, p=0.896, (-)	
Total water samples N = 123						
	Culture	Positive	19	29	36	12
		Negative	3	72	24	51
		Conformity	74.0%		71.5%	
		Significance	χ^2 = 22.87, p<0.00001, +++		χ^2 = 19.97, p<0.00001, +++	

pos. = positive; neg. = negative; (-) = conformity not significant; + = conformity significant at the level p<0.05; ++ = conformity significant at level p<0.01; +++ = conformity significant at level p<0.001.

also highly significant for total samples. No significant correlation between culture and both stages of semi-nested PCR was found for samples of cold water (Tab. 2).

Relationship between results obtained by culture and semi-nested PCR, depending on *Legionella* species. A highly significant correlation (p<0.01) was found between the occurrence of pure cultures of *Legionella pneumophila* serogroups 2-14 and the results of both stages of semi-nested PCR (Tab. 3). Unexpectedly, the association of *L. pneumophila* sg. 2-14 with strains of *L. pneumophila* sg. 1 and *Legionella* spp. resulted in a lack of significant correlation, except for a relationship between the culture pattern '*L. pneumophila* sg. 1 plus *L. pneumophila* sg. 2-14' and the first stage of PCR, where only a low significant correlation was noted (Tab. 3).

Correlation between concentration of *Legionella* in water determined by culture and results of semi-nested PCR. The concentration of *Legionella* in the examined water samples

Table 3. Correlation between detection of *Legionella* by culture and PCR, depending on species composition of *Legionella* in water samples

Source of water, species Total number of samples = 123		Type of PCR reaction			
		PCR 1 pos.	PCR 1 neg.	PCR 2 pos.	PCR 2 neg.
Culture: *L. pneumophila* sg. 1 + *L. pneumophila* sg. 2-14 + *Legionella* spp. CP=9	Positive	2	7	6	3
	Negative	20	94	54	60
	Conformity	78.0%		53.7%	
	Significance	χ^2 = 0.01, p=0.914, (-)		χ^2 = 0.59, p=0.442 (-)	
Culture: *L. pneumophila* sg. 1 + *L. pneumophila* sg. 2-14 CP=7	Positive	4	3	5	2
	Negative	20	96	54	62
	Conformity	81.3%		54.5%	
	Significance	χ^2 = 4.39, p=0.036, +		χ^2 = 0.79, p=0.374, (-)	
Culture: *L. pneumophila* sg. 2-14 + *Legionella* spp. CP=7	Positive	2	5	6	1
	Negative	20	96	53	63
	Conformity	79.7%		56.1%	
	Significance	χ^2 = 0.06, p=0.801, (-)		χ^2 = 2.79, p=0.095, (-)	
Culture: *L. pneumophila* sg. 2-14 CP=25	Positive	13	12	19	6
	Negative	9	89	41	57
	Conformity	82.9%		61.8%	
	Significance	χ^2 = 22.03, p<0.0001, +++		χ^2 = 7.99, p=0.0047, ++	

CP = total number of culture-positive samples at this species composition; pos.= positive; neg. = negative;
(-) = conformity not significant; + = conformity significant at level p<0.05; ++ = conformity significant at level p<0.01; +++ = conformity significant at the level p<0.001.

varied between 0-200+ per 100 ml, median was 0.00/100 ml and distribution was non-parametric (data not shown). By the use of Spearman's rank order test, a highly significant correlation was found to exist between the concentration of *Legionella* determined by culture and results of the semi-nested PCR, both for stage 1 (R=0.492, p<0.00001) and stage 2 (R=0.430, p<0.00001).

DISCUSSION

The study shows that the frequency of *Legionella* isolation from the urban MWSS hot water was strikingly greater than in rural cold water distributed by MWSS or taken from wells, either immediately, or by private water supply systems (PWSS). Although it is commonly known that *Legionella* strains are associated with hot water, the differences between hot and cold water found in this study are much greater than in our earlier work [2], where the isolation frequency of *Legionella* from urban and rural sources was comparable.

The frequency of the occurrence of *Legionella* determined by semi-nested PCR compared to culture was greater, on the borderline of significance. Nevertheless, statistical analysis showed a high correlation between the results of isolation by culture and DNA determination by the second stage of semi-nested PCR for warm water samples, while for cold water samples no significant correlation could be found. In this respect, our findings do not conform to those obtained by Lee *et al.* [10] who reported that in hot and cold water, culture and quantitative PCR gave similar results. The results of the presented study are rather in accord with those reported by Joly *et al.* [6] and Yaradou *et al.* [7] who found a significant correlation between culture and PCR results for hot water samples, and lack of correlation for cooling tower samples. Our results also conform with the data of Guillemet *et al.* [9] who found only a weak correlation between culture and real-time PCR in spa water. The significance of the correlation between these two methods noted in our study for hot water samples (p<0.001) is similar to that reported by Wellinghausen *et al.* [4].

In conclusion, our results support the opinion expressed by Szénási *et al.* [5] and Morio *et al.* [8] that though PCR cannot be a substitute for the isolation of *Legionella* by culture, it could be regarded as a useful complementary method.

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