Detection of *Legionella* spp. and occurrence of virulence genes: *lvh*, *rtxA* and *enhC* in water samples from artificial water systems

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation,
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▌**Abstract**
Introduction and objective. *Legionella* spp. are ubiquitous worldwide in natural water sources (rivers, lakes) as well as in man-made water systems (cooling towers, tap water, spas). The most common species causing the disease in humans is *Legionella pneumophila*. Pathogenicity of *Legionella* is related to the genes associated with virulence. The study aimed to detect *Legionella* spp. DNA in man-made water systems, and to examine the presence of selected virulence genes (*lvh*, *rtxA*, *enhC*) in *Legionella*-positive samples.
Materials and method. A total of 52 water samples from hot and cold water systems collected from urban and rural areas were investigated for the presence of *Legionella* by polymerase chain reaction (PCR). The detection of three virulence loci (*lvh*, *rtxA*, and *enhC*) in water samples was investigated using PCR.
Results. *Legionella* spp. was detected in over half the tested samples (55.77%), both in water from hot (55.88%) and cold (55.56%) water systems. Sequencing confirmed the presence of *Legionella pneumophila* in the tested samples. Among all *Legionella*-positive isolates, at least one virulence gene was detected in the case of 55.17% (16/29) of environmental samples. The *lvh* locus was most frequently present in all specimens.
Conclusions. The presence of *Legionella pneumophila*, the causative agent of Legionnaires' disease, in hot and cold water systems from rural and urban localities, indicates a risk to public health. The occurrence of virulence genes in tested samples suggests that *L. pneumophila* has the potential to cause human disease.

▌**Key words**
water, *Legionella*, *Legionella pneumophila*, environmental surveillance, virulence genes

INTRODUCTION

Members of the genus *Legionella* are intracellular, Gram-negative, waterborne pathogenic bacteria of great public health concern. The *Legionella* species are the etiologic agents of legionellosis [1]. Two clinical forms of the diseases are distinguished: severe atypical pneumonia called Legionnaires' disease, and the mild flu-like Pontiac fever, which generally ends with spontaneous recovery. Legionnaires' disease is an acute, multi-system infectious disease, most often with dominant symptoms of pneumonia. The mortality rate of Legionnaires' disease varies from 1%-12%, and may even be higher in risk groups (including immunocompromised patients) [2]. In 2010 in Europe, there was an increase in the number of cases of Legionnaires' disease from 6,255 to 11,307 in 2019 [3]. The great majority of human infections (approximately 90%) were caused by *Legionella pneumophila* [4].

Different species of *Legionella* ubiquitously inhabit natural water systems (rivers, lakes, streams, ponds) and can be also isolated from soil (*L. longbeachae*) [5, 6]. *Legionella* bacteria are commonly found in a wide range of artificial water systems, including cooling water towers, whirlpool spas, showers, fountains, pools, conditioning systems, and dental devices [7]. Human infection occurs by inhalation of aerosol from a bacteria-contaminated aquatic environment, or by aspiration of contaminated water from an environment [8]. In the human body, the inhaled bacteria enter the lungs, where it replicates in alveolar macrophages [9]. The ability of *Legionella* to cause the disease is related to the presence or absence of certain virulent genes. These genes may determine the survival of bacteria in the environment and the capacity to enter and replicate in host cells. To-date, many pathogenic loci have been identified, of which '*L. pneumophila* vir homologues' (*lvh*) and repeats in structural toxin (*rtxA*) are more frequently associated with the disease [10]. The *lvh* region encoding a type IVA secretion system (T4ASS) is related to the survival of the bacteria in the environment and contributes to virulence [11]. The *rtxA* gene has been shown to affect adherence and entry into human cells and intracellular survival and trafficking of the bacteria [12]. An important factor that contributes to the pathogenicity of bacteria is *L. pneumophila enhC* locus. The *enhC* gene encodes periplasmic protein EnhC, which is uniquely required for bacterial replication within macrophages. Thus, the presence of genes encoding virulence factors among *Legionella* isolates from a man-made water environment may provide valuable information about the pathogenic properties of the bacteria.

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OBJECTIVE

The aim of the study was (1) to detect *Legionella* spp. DNA in man-made water systems, which constitute an artificial reservoir of the bacteria, and (2) to examine the infection potential of *Legionella* by the detection of 3 selected virulence genes (*lvh*, *rtxA*, *enhC*) in *Legionella*-positive samples.

MATERIALS AND METHODS

Sample collection and DNA extraction. A total of 52 samples of drinking water were collected from hot and cold water systems. In detail, 34 samples were collected from hot-tap water systems from apartments within the city of Lublin, and 18 samples were collected from cold-tap water systems from houses in rural areas located in the Lublin Province. One hundred milliliters of water sample was collected from each site into sterile bottles and concentrated via membrane filtration using cellulose filters (0.45 µl pore size, Millipore). After filtration, membranes were scraped and the collected material suspended in 4 ml of phosphatase-buffered saline (PBS). The suspensions were transferred into sterile centrifuge tubes, then centrifuged at 5,000×g for 10 min. Subsequently, the supernatant was removed, and the remaining pellet suspended in 20–30 µl PBS for further analyses. The genomic DNA was isolated using commercial kit QIAamp DNA Mini Kit (Qiagen, Germany), following the kit protocols. DNA extracts were stored at –20 °C for further study.

PCR detection of *Legionella* and the virulence genes. Detection of *Legionella* was performed using the polymerase chain reaction (PCR) based on the fragment of the 16S rRNA gene, according to the procedure described previously by Wójcik-Fatla et al. [14]. Genomic DNA from *L. pneumophila* serogroups 2–14 isolated, identified by culture and Legionella Latex Test Kit (Oxoid, Basingstoke, Hampshire, UK), was used as a positive control [14]. The negative control was nuclease-free water. All *Legionella*-positive samples were examined for the presence of 3 virulence loci (*lvh*, *rtxA*, *enhC*) by PCR method. The 3 pairs of primers (lvh1/*prpA*-lvh2/*prpA*, lvh3/*lvhB3*-lvh/*lvhB4*, lvh5/*lvhB8*-lvh6/*lvhB9*) were used for amplification of the *lvh* region, the 2 pairs of primers (rtx1/*rtxA*-rtx2/*rtxA*, rtx3/*rtxA*-rtx4/*rtxA*) were used for the detection of the *rtxA* region, and the one pair of primers (enh1/*enhC*-enh2/*enhC*) was used for amplification of the *enhC* gene. The PCR reaction mixture and conditions were based on the methodology described by Samrakandi et al. [10]. The primers used for PCR analysis are summarized in Table 1.

DNA sequencing. The 16S rRNA PCR products were sequenced in both directions using primers JFP and JRP (Tab. 1) with a BigDye® Terminator v3.1 Cycle Sequencing Kit, then purified using Big Dye XTerminator Purification Kit (Applied Biosystems, USA), and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The nucleotide sequences were compared with data stored in the genetic sequence database (GenBank) using the Basic Local Alignment Search Tool (BLAST).

Table 1. Primers used in the study.

Name/region	Size	Sequence (5´–3´)	References
JFP	386 bp	AGGGTTGATAGGTTAAGAGC	[14]
JRP		CCAACAGCTAGTTGACATCG	
lvh1/*prpA*	260 bp	GTTTTAATCCCCCAGCAAGC	
lvh2/*prpA*		AATATCCCTACTCATCCTCG	
lvh3/*lvhB3*	1.0 kb	GGCTAGGAGGTTCTTGTG	
lvh4/*lvhB4*		ATTGGCCGAGATGTCCTT	
lvh5/*lvhB8*	280 bp	CCTCTACGCATTACAACGCC	
lvh6/*lvhB9*		GTGGTGGTAAAGGGAATGCC	[10]
rtx1/*rtxA*	630 bp	GATCCGCAAGTAGCGCTCAC	
rtx2/*rtxA*		TGTAATGCTGGCATTAGGCG	
rtx3/*rtxA*	540 bp	CTGATGCTGCTACGGAACAC	
rtx4/*rtxA*		CCGCAGTCATTACACCTGCG	
enh1/*enhC*	438 bp	AATGCTTTGTATGCCCTCGG	
enh2/*enhC*		CATATCAGCGCTTTGGCCATC	

RESULTS

A total of 52 drinking water samples from hot and cold water systems were tested. DNA of *Legionella* spp. was detected in 29 (55.77%) samples (Tab. 2). From the 34 water samples taken from the taps in the city of Lublin (hot water), bacterial DNA was detected in more than half of the samples (55.88%). A similar result was obtained for isolates from cold water taps collected from rural areas of the Lublin Province. Analysis of the 16S rRNA gene sequence fragment confirmed the presence of *L. pneumophila* in positive samples. BLAST analysis of the sequence of the 16S rRNA gene showed a percentage of similarity of 100% with sequences isolated from the environment (GenBank Acc. No. LC491286 – environmental water samples, and GenBank Acc. No. MH412937 – urban air sample), as well as from humans (GenBank Acc. No. CP021281).

In this study, to estimate whether *Legionella* inhabiting the tested water systems could cause human disease, the presence of 3 virulence loci was tested by PCR. Among all *Legionella*-positive isolates, at least one virulence gene (*lvh* or *rtxA* or *enhC*) was detected in 16 of the 29 (55.17%) samples. In the rural and urban samples, at least one virulence loci was found in 60.0% (6/10) and 52.63% (10/19), respectively. The most frequently detected gene in all tested isolates was the *lvh* loci (11/29). In both urban and rural samples, the *lvh* locus was predominant. The simultaneous presence of 3 pathogenic loci was found in 3 (10.34%) of all tested samples. In 13 samples (44.82%), the presence of the studied loci was not found. Results of the detection of virulence genes are shown in Table 2.

DISCUSSION

The study demonstrates that *Legionella* spp. is commonly present in hot and cold water systems in rural and urban areas. The new molecular techniques, such as the PCR method, enable *Legionella* spp. detection directly from the concentrated water samples. The DNA sequencing performed in this study confirmed the presence of *L. pneumophila*. In

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Table 2. Occurrence of *Legionella* spp. in hot and cold water systems from rural and urban localities, and detection of genes related to virulence (A – total of virulence loci detected, B – presence of 1, 2 or 3 loci)

Source of samples	No. of isolates	*Legionella*-positive samples (%)	A			B							
			lvh	*rtxA*	*enhC*	*lvh*	*rtxA*	*enhC*	*lvh+rtxA*	*lvh+enhC*	*enhC+rtxA*	*lvh+rtxA+enhC*	VG negative
						No. of detected loci/*Legionella*-positive samples (%)							
Rural (cold-water)	18	10/18 (55.56)	4/10 (40.0)	3/10 (30.0)	3/10 (30.0)	3/10 (30.0)	0/10 -	0/10 -	0/10 -	0/10 -	2/10 (20.0)	1/10 (10.0)	4/10 (40.0)
Urban (hot-water)	34	19/34 (55.88)	7/19 (36.84)	3/19 (15.79)	6/19 (31.58)	4/19 (21.05)	0/19 -	2/19 (10.53)	0/19 -	1/19 (5.26)	1/19 (5.26)	2/19 (10.53)	9/19 (47.37)
Total	**52**	**29/52 (55.77)**	**11/29 (37.93)**	**6/29 (20.69)**	**9/29 (31.03)**	**7/29 (24.14)**	**0/29 -**	**2/29 (6.9)**	**0/29 -**	**1/29 (3.45)**	**3/29 (10.34)**	**3/29 (10.34)**	**13/29 (44.83)**

VG – virulence gene

all examined samples, the occurrence of bacteria exceeded 55%. A significantly higher percentage of *L. pneumophila* incidence in public building water systems in the city of Lublin was found by Sikora et al. [15]. Bacteria were detected in 74.77% of samples from hot water systems (166/222). Simultaneously, in cold water systems the presence of bacteria was not detected. Significantly, *L. pneumophila* was present in over 78% of samples collected in hospitals (132/168) [15]. Wojtyła-Buciora et al. [16]. detected *Legionella* in 71% of samples taken from the hot water systems of public buildings, with 49% of samples from hospitals exceeding the acceptable level of *Legionella* contamination. Samples from other public buildings, e.g. hotels, welfare houses, and students hostels, presented a lower level of contamination (11–40%).

The environmental surveillance of water samples conducted by Napoli et al. [17] in Italy, showed similar results, where *Legionella* contamination was much higher in health care facilities (79.1%) than in community buildings (44.7%). Moreover, the prevalence of bacteria in water samples from apartments (54%) was similar to the presented study results (55.77%). Żak et al. [18] obtained a lower percentage of positive results than in the current study. They tested 366 samples from hot water systems of 111 teaching and education institutions in the Małopolskie Province, of which *Legionella* was present in 33.6%. Pierre et al. [19] examined 269 samples from domestic cold and hot water systems in 28 different buildings and from 45 recirculating hot water systems. *Legionella* was cultured from 24.2% (65/269) samples, which is half the percentage of positive results than in the current study.

The more frequent presence of *Legionella* in hospital water systems poses a risk of nosocomial infection, considering that hospitalized patients are more susceptible to infection. Farnham et al. [20] analyzed 1,449 legionellosis cases, of which 88.2% concerned patients with an underlying medical condition, which is a risk factor for legionellosis. On the other hand, clinical surveillance of 97 cases of legionellosis showed that 88 of them had community origin, while only 9 had nosocomial origin [17]. The current results and data from other countries [21, 22, 23] indicate that the microbiological contamination of water in apartments, private buildings, and individual households can be a source of infections.

It has been shown that differences in the ability to cause the disease between *Legionella* strains are related to the virulence genes. The study results show that out of 29 *Legionella*-positive samples, 16 harboured at least one of the tested virulent genes. The most prevalent locus in examined isolates was the *lvh* locus (37.93%). The *enhC* and *rtxA* loci

were presented in 31.03% and 20.69% of *Legionella*-positive isolates, respectively. The *lvh* and *rtxA* loci are predominantly present in *L. pneumophila* strains related to disease [10, 24]. The *enhC* locus is involved not only in the pathogenesis, but is also essential for the survival of bacteria in the environment [10]. The *lvh* and *rtxA* loci were much more often detected in water samples taken from hospitals in India than in the presented study. Out of 47 *L. pneumophila* isolates, both loci were present with a frequency of 97.8% [25]. Similarly, Katsiaflaka et al. [26] found pathogenicity genes *lvh* and *rtxA* in 93.8% and 95% of strains from environmental samples (hotels, cruise ships, ferries, and athletic venues) in Greece. The presence of both loci was detected in 91.3% of strains [26]. Hwang et al. [27] observed that 50% of environmental isolates collected in South Korea from cooling towers, hot and cold water systems from public facilities (public baths, large buildings, hotels, hospitals, factories, ships), carried 3 virulence genes (*dot/icm*, *lvh*, and *rtxA*).

The results obtained in the current study regarding the frequency of virulence genes in tested samples are lower than in the publications cited above. This may be due to the methodology used, where DNA was isolated directly from water samples without culture. The PCR amplification products of environmental samples showed lower intensity compared to the culture controls. Further research is required to assess the occurrence of virulence genes in *L. pneumophila* isolates from the environment on a larger samples size, also using the culture-based method.

Finally, the study design has some limitations. The PCR-based detection of *Legionella* may result in more positive outcomes than the standard culture-based method. PCR-based methods may detect DNA of not only viable but also dead bacteria, and have higher sensitivity [28]. On the other hand, PCR-based methods allow the detection of bacteria in a viable but non-culturable (VBNC) state. *L. pneumophila* at VBNC state loses the capability for growth on standard media, and at the same time still shows metabolic activity. Furthermore, VBNC cells are still able to produce proteins, which are also virulence factors [29]. Dietersdorfer et al. [30] confirmed that starved VBNC *Legionella* strains can infect human macrophage cell types. A decisive disadvantage of standard culture is the long waiting time for the result, even up to 10 days. Although culture remains the gold standard for bacteria investigation in the environment, new protocols based on real-time PCR assays to detect and quantify *Legionella* in water samples are proposed in order to improve and accelerate the bacteria detection process, especially in an outbreak situation [28, 31].

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CONCLUSIONS

The obtained results indicate that hot and cold water systems from rural and urban localities may be the source of *L. pneumophila* infection. The presence of virulence factors in the tested samples suggests that *L. pneumophila* has the potential to cause human disease. Although epidemiological data show that the number of cases of legionellosis in Poland does not exceed 100 cases per year [32], the risk of exposure to *Legionella* could be underestimated. Comprehensive water resource management programs should be implemented to prevent the growth and spread of *Legionella* in water systems supplying both public buildings, apartments and houses. The strategy should include routine testing of hot and cold water systems for *Legionella*, clear cleaning and disinfection procedures, controlling and maintaining the temperature of cold and hot water at an appropriate level (<20 °C for cold and ≥55 °C for hot water), preventing water stagnation in pipes (which promotes the formation of biofilms), and modernization of installations using materials that limiting *Legionella* colonization and biofilms formation.

REFERENCES

1. Cunha BA, Burillo A, Bouza E. Legionnaires' disease. Lancet. 2016; 387(10016): 376–385. https://doi.org/10.1016/S0140-6736(15)60078-2
2. Phin N, Parry-Ford F, Harrison T, et al. Epidemiology and clinical management of Legionnaires' disease. Lancet Infect Dis. 2014; 14(10): 1011–1021. https://doi.org/10.1016/S1473-3099(14)70713-3
3. The European Legionnaires' Disease Surveillance Network; The European Centre for Disease Prevention and Control. Surveillance Atlas of Infectious Diseases. Available online: https://atlas.ecdc.europa.eu/public/index.aspx (access: 2021.10.03).
4. Miyashita N, Higa F, Aoki Y, et al. Distribution of Legionella species and serogroups in patients with culture-confirmed Legionella pneumonia. J Infect Chemother. 2020; 26(5): 411–417. https://doi.org/10.1016/j.jiac.2019.12.016
5. Zhan XY, Zhu QY. Molecular typing of Legionella pneumophila isolates from environmental water samples and clinical samples using a five-gene sequence typing and standard Sequence-Based Typing. PLoS One. 2018; 13(2): e0190986. https://doi.org/10.1371/journal.pone.0190986
6. Potočnjak M, Magdalenić Z, Dijan M, et al. Environmental factors affecting the survival of soil dwelling Legionella longbeachae in water. Ann Agric Environ Med. 2016; 23(3): 452–455. https://doi.org/10.5604/12321966.1219186
7. van Heijnsbergen E, Schalk JA, Euser SM, et al. Confirmed and potential sources of Legionella reviewed. Environ Sci Technol. 2015; 49(8): 4797–4815. https://doi.org/10.1021/acs.est.5b00142
8. Prussin AJ, Schwake DO, Marr LC. Ten questions concerning the aerosolization and transmission of Legionella in the built environment. Build Environ. 2017; 123: 684–695. https://doi.org/10.1016/j.buildenv.2017.06.024.
9. Khweek AA, Amer A. Replication of Legionella pneumophila in human cells: Why are we susceptible?. Front Microbiol. 2010; 1: 133. https://doi.org/10.3389/fmicb.2010.00133
10. Samrakandi MM, Cirillo SL, Ridenour DA, et al. Genetic and phenotypic differences between Legionella pneumophila strains. J Clin Microbiol. 2002; 40(4): 1352–1362. https://doi.org/10.1128/JCM.40.4.1352-1362.2002.
11. Bandyopadhyay P, Liu S, Gabbai CB, et al. Environmental mimics and the Lvh type IVA secretion system contribute to virulence-related phenotypes of Legionella pneumophila. Infect Immun. 2007; 75(2): 723–735. https://doi.org/10.1128/IAI.00956-06
12. Cirillo SLG, Yan L, Littman M, et al. Role of the Legionella pneumophila rtxA gene in amoebae. Microbiology. 2002; 148(Pt 6): 1667–1677. https://doi.org/10.1099/00221287-148-6-1667
13. Liu M, Conover GM, Isberg RR. Legionella pneumophila EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. Cell Microbiol. 2008; 10(9): 1906–1923. https://doi.org/10.1111/j.1462-5822.2008.01180.x
14. Wójcik-Fatla A, Stojek NM, Dutkiewicz J. Efficacy of the detection of Legionella in hot and cold water samples by culture and PCR. I. Standardization of methods. Ann Agric Environ Med. 2012; 19(2): 289–293.
15. Sikora A, Wójtowicz-Bobin M, Kozioł-Montewka M, et al. Prevalence of Legionella pneumophila in water distribution systems in hospitals and public buildings of the Lublin region of eastern Poland. Ann Agric Environ Med. 2015; 22(2): 195–201. https://doi.org/10.5604/12321966.1152064
16. Wojtyła-Buciora P, Chrzanowska E, Marcinkowski JT. Presence of Legionella sp. in hot water systems of health care institutions and public buildings. Hygeia Public Health 2013; 48(3): 327–332.
17. Napoli C, Fasano F, Iatta R, et al. Legionella spp. and legionellosis in southeastern Italy: disease epidemiology and environmental surveillance in community and health care facilities. BMC Public Health. 2010; 10: 660. https://doi.org/10.1186/1471-2458-10-660
18. Żak J, Orlińska K, Koperny M, et al. Legionella sp. in water systems in public teaching and education facilities in Małopolskie voivodeship in 2016. Przegl Epidemiol. 2019; 73(2): 227–237. https://doi.org/10.32394/pe.73.21
19. Pierre D, Baron JL, Ma X, et al. Water quality as a predictor of Legionella positivity of building water systems. Pathogens. 2019; 8(4): 295. https://10.3390/pathogens8040295
20. Farnham A, Alleyne L, Cimini D, et al. Legionnaires' disease incidence and risk factors, New York, New York, USA, 2002–2011. Emerg Infect Dis. 2014; 20(11): 1795–1802. https://doi.org/10.3201/eid2011.131872
21. Lampl BMJ, Lang M, Wodnick S. Can mandatory monitoring in rental apartments effectively prevent legionellosis? A retrospective analysis of data from Regensburg with a review of the literature. GMS Hyg Infect Control. 2020; 15: Doc14. https://doi.org/10.3205/dgkh000349
22. Ricci ML, Rota MC, Caporali MG, et al. A Legionnaires' disease cluster in a private building in Italy. Int J Environ Res Public Health. 2021; 18(13): 6922. https://doi.org/10.3390/ijerph18136922
23. Hayes-Phillips D, Bentham R, Ross K, et al. Factors influencing Legionella contamination of domestic household showers. Pathogens. 2019; 8(1): 27. https://doi.org/10.3390/pathogens8010027
24. Huang B, Heron BA, Gray BR, et al. A predominant and virulent Legionella pneumophila serogroup 1 strain detected in isolates from patients and water in Queensland, Australia, by an amplified fragment length polymorphism protocol and virulence gene-based PCR assays. J Clin Microbiol. 2004; 42: 4164–4168. https://doi.org/10.1128/JCM.42.9.4164-4168.2004
25. Sreenath K, Chaudhry R, Vinayaraj EV, et al. Distribution of virulence genes and sequence-based types among Legionella pneumophila isolated from the water systems of a tertiary care hospital in India. Front Public Health. 2020; 8: 596463. https://doi.org/10.3389/fpubh.2020.596463
26. Katsiaflaka A, Pournaras S, Kristo I, et al. Epidemiological investigation of Legionella pneumophila serogroup 2 to 14 isolates from water samples by amplified fragment length polymorphism and sequence-based typing and detection of virulence traits. Appl Environ Microbiol. 2016; 82: 6102–6108. https://doi.org/10.1128/AEM.01672-16
27. Hwang IY, Park EH, Lee YC. Distribution of virulence genes in environmental and clinical isolates of Legionella pneumophila in Busan, South Korea. Southeast Asian J Trop Med Public Health. 2017; 48(1): 83–90.
28. Toplitsch D, Platzer S, Zehner R, et al. Comparison of updated methods for Legionella detection in environmental water samples. Int J Environ Res Public Health. 2021; 18(10): 5436. https://doi.org/10.3390/ijerph18105436
29. Alleron L, Khemiri A, Koubar M, et al. VBNC Legionella pneumophila cells are still able to produce virulence proteins. Water Res. 2013; 47(17): 6606–6617. https://doi.org/10.1016/j.watres.2013.08.032
30. Dietersdorfer E, Kirschner A, Schrammel B, et al. Starved viable but non-culturable (VBNC) Legionella strains can infect and replicate in amoebae and human macrophages. Water Res. 2018; 141: 428–438. https://doi.org/10.1016/j.watres.2018.01.058
31. Collins S, Jorgensen F, Willis C, et al. Real-time PCR to supplement gold-standard culture-based detection of Legionella in environmental samples. J Appl Microbiol. 2015; 119(4): 1158–1169. https://doi.org/10.1111/jam.12911
32. National Institute of Public Health-National Institute of Hygiene, 2021. Reports on cases of infectious diseases and poisonings in Poland. http://www.pzh.gov.pl (access: 2021.10.03).