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Occurrence of *Legionella* spp. in water samples from selected basins in the Lesser Poland (Southern Poland) and identification of the main serogroups

Abstract

Legionella are small gram-negative rod-shaped bacteria. Some species occur in the environment and are not pathogenic while the species *Legionella pneumophila* cause the majority of human infections. *Legionella* are relatively resistant to standard water disinfection procedures, and, can occur in potable water. They can also survive within biofilms formed in aquatic systems. Sporadic and epidemic cases of legionellosis occur all over the world. *Legionella* are transmitted directly from the environment to humans. Infection results from exposure to contaminated aerosols, e.g. under showers, fountains, swimming pools, swimming pool basins, air humidifiers, and air conditioning, hot water installations in car washes. *L. pneumophila* is divided into 15 serogroups, among which serogroup 1 is the most prevalent disease-causing variant. The aim of this work was to present the frequency of *Legionella* occurrence in water samples collected in the period of October to December 2021 from selected swimming pools in Lesser Poland and to identify the main serogroups of *Legionella* spp., which may be dangerous due to their pathogenicity.

Keywords: gram-negative bacilli, pathogenicity, swimming pool water, serotypes

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Introduction

The genus *Legionella* Brenner has 58 species and 3 subspecies (Yu et al., 2002). About half of the species of the *Legionella* genus can cause disease in humans, while the rest occur in the environment and are not pathogenic. *Legionella pneumophila* Brenner et al. parasitises in cells of phylogenetically distant hosts, in the aquatic environment in protozoa, and in the human body in alveolar macrophages (Nisar et al., 2020).

The ability of these bacteria to multiply intracellularly in phagocytic cells, specialized to destroy microorganisms, is of fundamental importance for the development of atypical pneumonia called Legionnaires' disease. The bacillus *L. pneumophila* was first isolated during a pneumonia epidemic in 1976 that affected members of the American Legions attending the congress in Philadelphia (Iliadi et al., 2022; Mondino et al., 2020).

Legionella bacilli are gram-negative, pleomorphic, and when observed in tissues, they look like cocci. However, in *in vitro* cultures they have various shapes and can reach a length of up to 20μm. The optimal growth temperature ranges between 25–43°C. These bacilli can survive even at temperatures of 55–60°C, as evidenced by frequent cases of their isolation from hot water distribution systems in public buildings and housing estates. *Legionella* is an obligate aerobe (Krause, 2022).

In breeding, these bacteria require media enriched with L-cysteine and iron. The lack of growth on blood agar is the basis for the preliminary identification of these bacteria. These bacteria have developed special mechanisms of iron uptake into the cell from the external environment, involving the secretion of the so-called siderophores, i.e. organic molecules that strongly bind iron ions and are taken back by the cell in the form of complex (coordination) compounds (Burnside et al., 2015; Cianciotto, 2015; Liles et al., 2000). Loss of this ability is generally associated with loss of virulence.

Legionella bacteria can survive in humid environments for long periods at relatively high temperatures and in the presence of disinfectants, including chlorine (Borella et al., 2016; Kim et al., 2002; Xi et al., 2024). They can also survive within biofilms formed in aquatic systems (Chauhan, Shames, 2021; Steinert et al., 2002). Sporadic and epidemic cases of legionellosis occur all over the world. Infection in humans results from exposure to contaminated aerosols, e.g. under showers, fountains, swimming pools, swimming pool basins, air humidifiers, and air conditioning, hot water installations in car washes.

Legionella pneumophila serogroup 1 was the predominant serogroup (84.2%), and serogroups 2–13 (7.4%) accounted for the remaining serogroups (Yu et al., 2002). The *Legionellaceae* Brenner et al. family contains one genus of *Legionella*. The number of species and serogroups is constantly increasing. There are 15 serogroups of *Legionella pneumophila* and two each for *L. bozemanae* Brenner et al., *L. longbeachae* McKinney et al., *L. feeleii* Herwaldt et al., *L. hackeliae* Brenner et al., *L. sainthelensi* Campbell et al., *L. spiritensis* Brenner et al., *L. erythra* Brenner et al., *L. quinlivanii* Benson et al., and one serogroup for the remaining species (Fields et al., 2002). Symptomatic infections caused by *Legionella* can occur in two forms: flu-like infection, i.e. the so-called Pontiac fever, and severe pneumonia, i.e. Legionnaires' disease. Microbiological tests are necessary to confirm the diagnosis (Rello et al., 2024).

The aim of this study was to present the frequency of *Legionella* occurrence in water samples collected in the next three months from October to December 2021 from selected swimming pools in Lesser Poland and to identify the main serogroups of *Legionella* spp., which may be disease-causing variant.

Materials and Methods

Quantification of *Legionella* bacteria in accordance with PN-EN ISO 11731: 2017-08

Quantification of Legionella spp. bacteria

Water samples (100 ml) were filtered twice, and the filters were incubated on GVPC agar medium (VWR International, selective medium buffered with charcoal, yeast extract, glycine, vancomycin, polymyxin B and cyclohexamide) with acidic buffer (0.2 M HCl + 0.2 M KCl). The filter was washed with Ringer's solution and then placed on GVPC agar medium and BCYE agar medium (VWR International, buffered agar medium with charcoal, yeast extract and cysteine). The plates were incubated at 36±2°C in a moist chamber. Reading was performed on the third, seventh and tenth days of incubation. A plate with presumed typical *Legionella* spp. colonies (white-grey colonies, smooth with a full rim with a characteristic ground glass appearance) (Fig. 1A) was inoculated onto BCYE and blood agar. On the tenth day, colony growth was assessed. A positive result was considered to be colonies growing on BCYE, and not growing on blood agar (Fig. 1B).

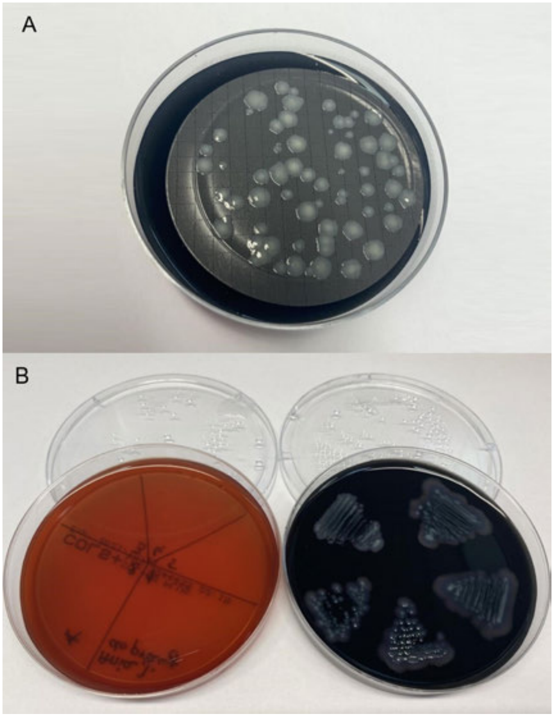

Fig. 1. Typical *Legionella* spp. colonies on GVPC medium – A, confirmed *Legionella* spp. colonies. Left – blood agar (no growth), right – BCYE (visible growth) – B (Photo. A. Jękot)

Identification of the major serogroups of *Legionella* spp. using the Legionella Latex Test

In order to identify *Legionella pneumophila* serogroup 1, *Legionella pneumophila* serogroup 2–14, and seven other most common pathogenic *Legionella* strains, a *Legionella* Latex Test (ThermoFisher Scientific, USA) was performed. A positive result was considered to be the appearance of agglutination in any of the fields with the test reagent within a maximum of 1 minute. A negative result was considered to be the absence of agglutination in all fields (Fig. 2).

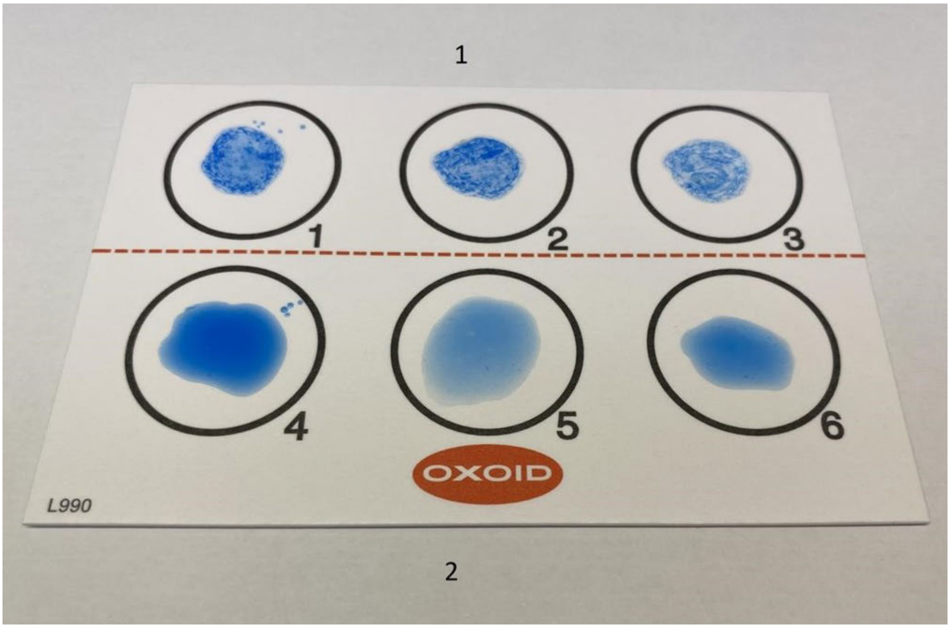

Fig. 2. Confirmed *Legionella* spp. serogroups; 1 (1, 2, 3) – visible agglutination, 2 (4, 5, 6) – no agglutination (Photo. A. Jękot)

Results

Frequency of occurrence of *Legionella* spp. in the tested swimming pool water

Water samples were taken from swimming pools and swimming pool equipment (swimming pool basin, showers, jacuzzi, circulating water, paddling pools, swimming pools) located at various facilities in Lesser Poland.

The number of confirmed *Legionella* spp. colonies was 75 occurrences out of 613 samples analysed during the period October – December 2021. The highest number of *Legionella* cases detected was in October, after which a decrease in positive results was observed in the following months (Tab. 1).

Tab. 1. Summary of the test results for the presence of *Legionella* spp. in swimming pool water in the period October-December 2021

Months	Number of tested samples	Confirmed *Legionella* spp. colonies		Alleged unconfirmed *Legionella* spp. colonies		Lack of growth	
		number	share [%]	number	share [%]	number	share [%]
October	255	35	14	72	28	149	58
November	214	25	12	64	30	125	58
December	144	15	10	37	26	92	64
Total number	613	75	12	173	28	367	60

A comparison of water samples taken from the pool and water pool equipment showed that in 48% of water samples *Legionella* spp. bacteria were detected in the swimming pool basin, 25% in the jacuzzi, 15% in circulating water, 7% in the shower tray, 2% in the men's shower and 1% in the women's shower (Fig. 3).

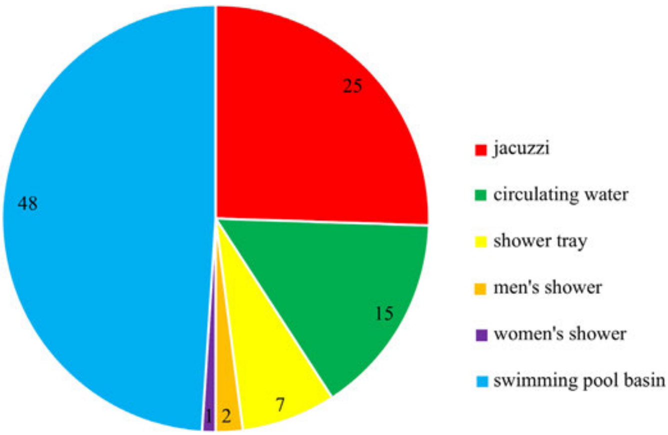

Fig. 3. Confirmed cases of *Legionella* spp. [%] in swimming pool water and various water pool equipment

Legionella spp. serogroups in the tested water

Using the Oxoid *Legionella* Latex Test, serotyping of randomly selected confirmed colonies of *Legionella* spp. was performed. The largest group, as many as 59%, was *Legionella pneumpohila* serogroups 2–14 (red), followed by 17%, the most pathogenic *Legionella pneumophila* serogroup 1 (blue), 12% was a group of additional *Legionella* strains, i.e. *L. longbeachae*, *L. bozemanae*, *L. dumoffii* Brenner et al., *L. gormanii* Morris et al., *L. jordanis* Cherry et al., *L. micdadei* Hébert et al., *L. anisa* Gorman et al. (green). In 12% of the colonies tested, no *Legionella* spp. strain was detected using the test (yellow) (Fig. 4).

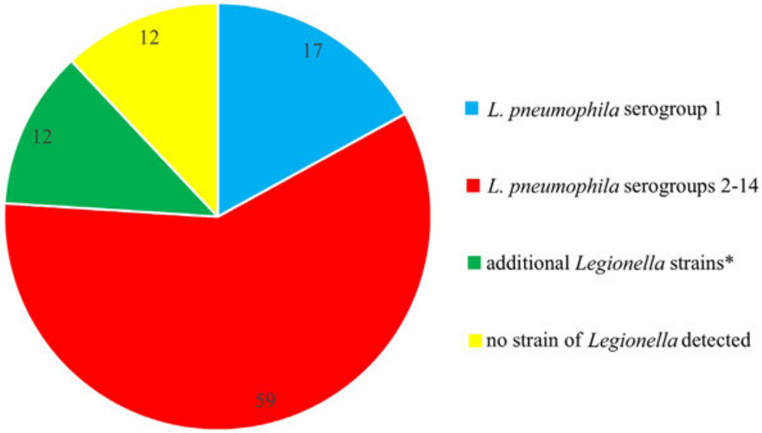

Fig. 4. Identification of major serogroups of *Legionella* spp. [%]; **L. longbeachae* McKinney et al. 1982, *L. bozemanae* Brenner et al. 1980, *L. dumoffii* Brenner et al. 1980, *L. gormanii* Morris et al. 1980, *L. jordanis* Cherry et al. 1982, *L. micdadei* Hébert et al. 1980, *L. anisa* Gorman et al. 1985

Discussion

Swimming pool water is a subject to the requirements regarding the number of *Legionella* bacteria, which were introduced by the Regulation of the Minister of Health (*Regulation of the Minister of Health of November 9, 2015 on the requirements to be met by water in swimming pools, OJ 2015 item 2016*). *Legionella* bacteria should not be detected in water samples with a volume of 100 ml coming from swimming pools equipped with devices generating water and air aerosol, swimming pools provided for swimming lessons for infants and small children up to 3 years of age, and from swimming pools provided the water at temperature equalling to or higher than 30°C. The same requirements also apply to water introduced into the pool basin from the circulation system. However, water in showers from hot water installations is subject to the same requirements as hot water in collective housing buildings. i.e. the number of these bacteria should be less than 100 CFU in a 100 ml water sample.

Water tests carried out in recreational and sports facilities showed the presence of *Legionella* in 75 water samples (out of 613 samples tested) collected in October, November and December. The greatest number of water samples indicating the presence of *Legionella* were detected in October, after which a decrease in the number of *Legionella* detections was observed in the following months.

The report of the Sanitary and Epidemiological Station in Poland shows that the number of *Legionella* infections is highest in the summer season due to the prevailing temperatures, after which a gradual decline in infections is noticed (Gług, 2024). Also ECDC (European Center for Disease Prevention and Control) observed that the peak of infections occurred in the summer season and then the gradual decrease was confirmed.

The results presented here show that these bacteria were most often detected in the swimming pool basin, in the jacuzzi, in circulating water, and in the swimming pool. The highest number of cases of *Legionella* spp. detected in the swimming pool basin is probably the results of the available water temperature, which ranges from 20–35°C. This is the optimal growth temperature for these bacteria (the optimal bacteria growth temperature range is 20–45°C), and also because swimming pools are used by a large number of people. The next place where *Legionella* spp. was detected is a jacuzzi. In this case the temperature is also a key factor, varying between 35 and 38°C. In circulating water, the main factor may be water flow systems, which are used to purify water that then goes to swimming pools. The water was probably improperly disinfected, and deposits were formed, which led to the formation of a biofilm, being also an ideal milieu for the growth of *Legionella* spp. bacteria. Improper disinfection of circulating water has a direct impact on the condition of the water in the swimming pool, where single cases of *Legionella* spp. were also detected. In the case of water from shower trays and showers, the reason for the formation of bacteria could be its transfer by people on their bodies and insufficient disinfection of the installation, which contributes to the creation of ideal conditions for the growth of bacteria.

In the years 2008–2016, the number of *Legionella* infections in Poland was relatively low, ranging from 8 to 36 cases per year. However, since 2017 there has been a marked increase in the number of cases. In 2019, the number of infections was approximately twice as high as the peak number of infections in 2008–2016. In 2022, the number of infections amounted to 118 cases. The culmination occurred in 2023, with as many as 240 infections. This is the highest number of infections recorded in the analyzed period (Gług, 2024). The described screening results concern the SARS-CoV-2 pandemia time. Groups at risk are largely the same for COVID-19 and Legionnaires' disease (LD). *L. pneumophila* is responsible for >90% of LD cases; especially, serogroup 1 causes 70%–80% of LD cases in Europe and the United States. Therefore, it crucial to make differential diagnoses during the COVID-19 pandemia by diagnostic microbiology to identify other infectious microorganisms, e.g. *Legionella* causing similar symptoms (Verhasselt et. al, 2021).

In August 2023 there occurred *Legionella* infection in Rzeszów (Podkarpackie Voivodeship). As a result of water contamination with this bacterium, over 170 people became ill and 25 people died. The State Sanitary Inspectorate of the Podkarpackie Voivodeship conducted an epidemiological investigation to identify the probable source of infection in the outbreak in Rzeszów and the surrounding area. As you can read in the ECDC report, in 2021, 9% of all cases of bacterial infections resulted in death (Annual Epidemiological Report for 2021).

The most frequently used methods of *Legionella* eradication are disinfection: thermal (e.g. raising the temperature of circulating water above 60°C for one minute), chemical (chlorination or ozonation) and increasing the ionic concentration of bactericidal metals (copper and silver) (Maynard, Whapham, 2020; Khan et al., 2021).

Unfortunately, most "classic" methods of combating *Legionella* are ineffective or expensive and their effect is short-lived. Large doses of chlorine are unacceptable for drinking water circuits. Water with a high concentration of chlorine compounds is very aggressive to the installation. Regular overheating of the installation leads to the formation of sediments, clogging of the installation and reducing the life of the hot water preparation technology. In addition, proper thermal disinfection is associated with increased costs of heat supply, and *Legionella* is only destroyed where the water temperature reaches over 70°C. Additionally, such hot water may be dangerous for recipients. Water disinfection with copper and silver electrodes is a long-term and uncertain process, suitable only for closed circuits (hot water) (*Zwalczanie Legionelli*…, legionella.pl).

In hospital facilities, in addition to constant monitoring of water quality, it is recommended to filter it and irradiate it with UV rays, especially in patients from the high-risk group (Maynard, Whapham, 2020; Khan et al., 2021). To reduce the risk of Legionnaires' disease, routine inspection and microbiological monitoring should be implemented to assess the presence of the most pathogenic species, namely *L. pneumophila* which may exist within the water system.

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Conflict of interest

The authors declare no conflict of interests.

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Zwalczanie Legionelli – jak można pozbyć się grożnej bakterii (legionella.pl), [access: 13.11.2024] [In Polish]

Występowanie bakterii *Legionella* spp. w próbkach wody z wybranych basenów Małopolski (Południowa Polska) i identyfikacja głównych grup serologicznych

Streszczenie

Legionella to małe bakterie Gram-ujemne w kształcie pałeczki. Niektóre gatunki występują w środowisku i nie są chorobotwórcze, natomiast gatunki *Legionella pneumophila* powodują większość infekcji u ludzi. *Legionella* jest stosunkowo odporna na standardowe procedury dezynfekcji wody i może występować w wodzie pitnej. Może również przetrwać w biofilmach utworzonych w systemach wodnych. Sporadyczne i epidemiczne przypadki legionellozy występują na całym świecie. *Legionella* przenoszona jest bezpośrednio ze środowiska na człowieka. Zakażenie następuje w wyniku narażenia na zanieczyszczone aerozole, m.in. pod prysznicami, przy fontannach, w basenach, nieckach basenowych, od nawilżaczy powietrza i klimatyzacji, instalacji ciepłej wody w myjniach samochodowych. *L. pneumophila* dzieli się na 15 serogrup, spośród których serogrupa 1 jest najbardziej rozpowszechnionym wariantem chorobotwórczym. Celem pracy było przedstawienie częstości występowania *Legionelli* w próbkach wody pobranych w okresie od października do grudnia 2021 roku z wybranych basenów małopolskich oraz identyfikacja głównych grup serologicznych *Legionella* spp., które mogą być niebezpieczne ze względu na swój charakter. patogeniczność.

Słowa kluczowe: gram-ujemne pałeczki, patogenność, woda basenowa, serotypy

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She is interested in broadly understood microbiology and protistology. In recent years, her research has focused on phylogenetic relationship among *Paramecium bursaria* Ehr. strains and endosymbiosis in *P. bursaria* (Ciliophora, Oligohymenophorea). The second research topic is searching for new compounds with antibacterial properties.

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