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Prevalence, Genetic Diversity, and Virulence Potential of *Legionella spp.*

Summary of the Doctoral Thesis for obtaining the scientific degree “Doctor of Science (*PhD*)”

Sector Group – Medical and Health Sciences
Sector – Basic Medicine
Sub-Sector – Other Basic Medicines

Riga, 2023

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The Doctoral Thesis is available at the RSU Library and on the RSU website: https://www.rsu.lv/en/dissertations

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Abbreviations used in the Thesis

cgMLST	Core Genome Multilocus Sequence typing
ECDC	European Centre for Disease Prevention and Control
EU	European Union
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESGLI	ESCMID Study Group for Legionella Infections
FLA	Free living amoeba
CFU	Colony forming units
LCV	*Legionella* containing vacuole
LLAP	*Legionella* like amoebal pathogens
MIF	Mature intracellular form
MK	Cabinet of Ministers
NGS	Next Generation Sequencing
PCR	Polymerase chain reaction
SBT	Sequence based typing
SG	Serogroup
SPKC	Centre for Disease Prevention and Control
VBNC	Viable but not culturable

Introduction

The wide range of infections caused by bacteria of *Legionella* spp., with manifestations varying from light fever (Pontiac fever) to acute and potentially lethal pneumonia (Legionnaires' disease) are currently referred to as legionellosis (Phin, 2014). Human cases of *Legionella* spp. infections typically occur through the inhalation of infected aerosols or via aspiration (Bollin et al., 1985). Legionellosis belongs to the class of sapronoses, infections caused by free-living organisms that can become the causative agents of infection and multiply in host organism under certain conditions. Humans are not the principal host organism for these bacteria (Kuris et al., 2014).

Legionnaires' disease is still a significant cause of preventable morbidity and mortality in Europe. The majority of Legionnaires' disease cases are sporadic, however, there are also several infection clusters and travel-related cases reported each year. The average reported occurrence in EU/EEA is 1.9–2.5 cases per 100 000 population per year. The occurrence in Latvia was 2.2 cases per 100 000 population in the year 2019, but increased to 4.5 cases per 100 000 population in the year 2021. Furthermore, over 85 % of all Legionnaires' disease cases were recorded in the capital city Riga and its suburbs, where the occurrence per 100 000 population in the year 2020 reached 6.0 and 4.5, respectively (ECDC, 2022; SPKC, 2023). Laboratory diagnostics of Legionnaires' disease is effective mainly in cases caused by serogroup 1 of *L. pneumophila*. The specificity and sensitivity of the available test methods against other serogroups and species is still far from ideal.

More than 60 species of *Legionella* are known, but the causative agent of Legionnaires' disease in more than 90 % of cases is *Legionella pneumophila*. *Legionella* are ubiquitous, they have been found in groundwater and surface waters, damp soils, but their principal reservoirs are artificial aqueous environments. The very low concentrations of *Legionella* in natural biotopes can

be greatly magnified in hot water supply pipes if the minimum temperature requirements for the control of opportunistic pathogens are not met by facility management (Singh et al., 2022). Contamination is directly associated with the circulating water temperature (Kruse et.al., 2016) and water stagnation may lead to the release of bacteria from biofilms upon changes in water flow (Nisar et al., 2020; Yu et al. 2019).

Humans are an accidental host for *Legionella* and the ability of these bacteria to infect humans is considered to arise from the long coevolution of bacteria with unicellular organisms. The same virulence factors enabling the infection of unicellular organisms by *Legionella* also play a role in the infection of human alveolar macrophages (Oliva et al., 2018). Free-living unicellular organisms provide *Legionella* with nutrients and additional shielding from such environmental factors as temperature and disinfectants (Shaheen et al., 2019).

The reasons for different virulence of specific *Legionella* species and strains against human hosts have not yet been fully elucidated, but the presence of certain genes encoding for virulence factors is widely suspected. The most prominent virulence factors of *Legionella* are associated with the bacterial cell wall structure, as well as systems for the secretion of effector proteins (Gattuso et al., 2022). Horizontal gene transfer from host amoebae has provided *Legionella* with many eukaryotic type genes that control the infection of host cells (Scheithauer et al., 2023). Several of the bacterial effectors can fulfil parallel functions in different organisms and some effector functions can overlap or substitute each other in pathogenic and non-pathogenic species of *Legionella* (Yang et al., 2023).

The risk of infection with Legionnaires' disease can be substantially mitigated with preventive measures, even though the source of infection cannot be completely eliminated. The development and implementation of water supply safety plans is a generally accepted practice, where the risks of every action are

weighted with regard to the risk of *Legionella* propagation. Such a plan provides a detailed and systematic determination and assessment of risks, creating justification for the control measures and inspection of facilities (ESGLI, 2017).

Aim of the Thesis

The aim of the thesis was to study the trends in *Legionella* occurrence, genetic diversity, and virulence potential for the diagnostics and prevention of legionellosis in Latvia.

Tasks of the Thesis were:

1. to determine *Legionella pneumophila* seroprevalence among blood donors in Latvia and the evaluation of factors associated with seropositive status;
2. to study prevalence of *Legionella* spp. and the effects of hot water temperature and the presence of free-living amoebae on the colonisation and persistence of *Legionella* spp. in water-supply systems;
3. to investigate the diversity of *Legionella pneumophila* cgMLST sequence types;
4. to identify virulence genes in *Legionella pneumophila* isolates and characterize their virulence potential.

Scientific hypothesis of the Thesis

It is proposed that the *L. pneumophila* strains persistent in water-supply systems can act as infectious agents with a high virulence factor regardless of their serogroup, sequence type, and the array of virulence factors at the moment of testing.

Novelty of the Thesis

This is the first study in Latvia demonstrating factors associated with *L. pneumophila* seropositivity, as well as the diversity and prevalence of various *Legionella* spp. serogroups and sequence types in water-supply systems. The results of this study demonstrated that the predominant *L. pneumophila* serogroups in Latvia are SG 2 and SG 3, and that the cases of Legionnaires' disease caused by these serogroups are not revealed by urine antigen test, which is the most commonly used diagnostic method both worldwide and in Latvia. During this study, ten previously unreported *Legionella* spp. sequence types were isolated and sequenced, contributing to the worldwide database of *Legionella* spp. genetic profiles.

1 Materials and methods

1.1 Study design and implementation

The study was planned as a sequence of three linked phases implemented from September 2013 until March 2023 (Figure 1.1). The collection of blood samples started in February 2014 after the initial preparatory phase, while processing of the last sequencing data was completed in March 2023.

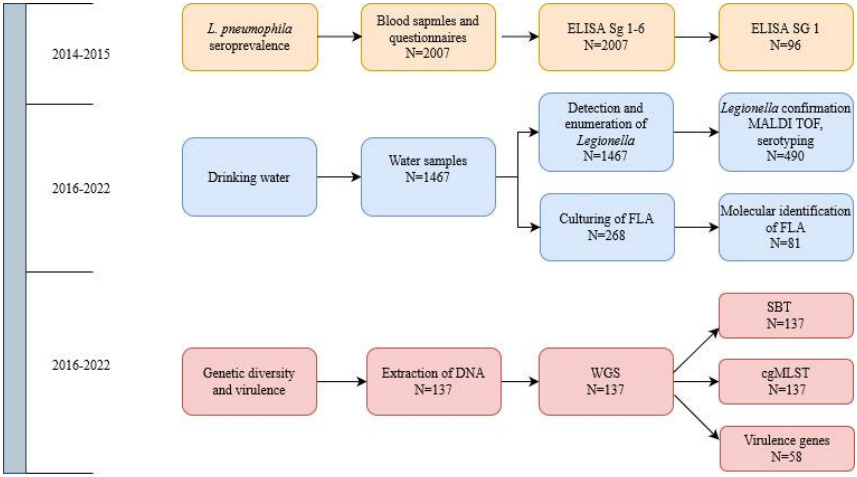

Figure 1.1 **Study timeline and methodology**

1.2 Study population and the collection of blood samples

A total of 2007 blood samples were collected from healthy blood donors throughout the territory of Latvia between February 2014 and October 2014 in cooperation with the Latvian State Blood Donor centre. The most recent population census data of 2011 were used for achieving appropriate coverage of the population (CSB, 2011). According to the population census of 2011, the adult population of Latvia was 1 250 000 and the coverage was planned at 0.15–0.20 % level of the adult population, therefore the study should cover between

1875 and 2500 participants. On the basis of national census data, the blood donors were selected proportionally to the sex and age in each region, forming a spreadsheet of planned sex and age distribution of blood donors for this purpose. This spreadsheet was used as a tool for the selection of study participants in each region of Latvia.

The specialists of the Latvian State Blood Donor centre recruited study participants from the available donors during each blood drive, in order to reach the target numbers given in Table 2.4. The study was approved by the Riga Stradiņš University Research Ethic Committee (Annex 1). Each donor was assessed before blood draw by a licenced physician. The participants were interviewed and asked to fill out a questionnaire in the presence of a Latvian State Blood Donor centre representative (Annex 2). The questionnaire included social and demographic parameters of donors, including age, sex, personal habits (smoking), place of residence, type and age of the housing, type of hot water supply system, water boiler, exposure to water aerosols at work, such as in car wash, spa, dental clinics, industrial settings, shower use outside of home, as well as the history of flu-like symptoms, pneumonia, and other respiratory illnesses during the preceding year.

1.3 Sampling of drinking water from water-supply systems

A total of 1467 water samples were collected, comprising 192 cold water samples and 1275 hot water samples from multi-apartment buildings, hotels, and other public buildings including gyms, offices, etc. The sampling plan included buildings receiving water from various sources – underground and treated surface waters. The samples were collected in Riga (1096 of 1467 samples) and in other Latvian cities or towns (371 of 1096 samples). Altogether, the selection covered 317 buildings, of which 204 were located in Riga and 113 in other areas of Latvia.

The sampling was performed in accordance with ISO 19458 (ISO 19458:2006). In each residential building, at least one hot water sample was collected from a shower head. Additional samples were collected depending on the size of the building and the response of residents, including a cold water sample from shower head and a hot water sample from faucet. In each hotel, the samples were collected from at least three locations, for example, mixer taps, shower heads in hotel suites, boiler rooms, gyms, changing rooms, and SPA facilities.

The water flow was allowed to run for at least three minutes before sample collection. In hotels, the samples were collected immediately after opening the faucet, as well as after three minutes, in order to assess water contamination with *Legionella* spp. both under stagnation and circulation conditions.

In accordance with the information provided by the building managers and the inhabitants, 132 multi-apartment buildings with previous history of Legionnaires' disease cases were known. There was no information about the rest of the buildings. All multi-apartment buildings included in this study were older than 30 years. Each drinking water sample was filled into a sterile 1L bottle and the cap was tightly closed. The water temperature was measured during sampling with a calibrated thermometer. A specially equipped vehicle was used for maintaining 0 °C to 6 °C temperature during the sample transportation from sampling sites to the testing laboratory. Sample testing was started no later than 6 hours after the sampling.

1.4 Serological methods

All 2007 blood samples were tested for both IgG and IgM antibodies against *L. pneumophila* SG 1–6, using indirect immunoassay method (*Vircell*, Granada, Spain). The testing of samples was repeated in the case of inconclusive

results. In accordance with the manufacturer's instructions, samples with index below 9 were assumed to contain no antibodies against *L. pneumophila*, while samples with index above 11 contained antibodies against *L. pneumophila*. All positive samples were additionally tested for *L. pneumophila* SG 1, using indirect immunoenzymatic method (*L. pneumophila* SG 1 ELISA IgG, *Vircell*, Granada, Spain).

1.5 Microbiological identification and enumeration of *Legionella*

One litre of water was filtered through a polyamide membrane filter (47 mm diameter) with 0.45 μm pore size (*Millipore*, Molsheim, France). The membrane filters were resuspended on a Petri dish with sterile, distilled water (5 mL) and vortexed for 2 min (*Vortex Genius*, IKA, Staufen, Germany), then maintained at room temperature for 10 min. The determination of *Legionella* spp. was performed in accordance with ISO 11731 (ISO 11731:2017). A total of three 0.1 mL aliquots of the sample (untreated, thermally treated, and acid-treated) were seeded on a buffered charcoal yeast extract (BCYE) agar growth medium (Oxoid, Basingstoke, United Kingdom) and on a glycin, vancomycin, polymixin B, cycloheximide (GVPC) agar growth medium (Oxoid, Basingstoke, United Kingdom), the plates were incubated at 36 ± 2 °C. At least three characteristic colonies from each plate were selected for subculturing on plates with buffered charcoal yeast extract (BCYE) agar growth medium with L- cysteine (Oxoid, Basingstoke, United Kingdom) and buffered charcoal yeast extract agar growth medium without L-cysteine (BCYE-Cys, Oxoid, Basingstoke, United Kingdom), followed by incubation for at least 48 h at 36 ± 2 °C temperature.

Legionella species were identified in suspected colonies with matrix-assisted laser desorption ionisation – time-of-flight mass spectrometry (*MALDI-TOF MS*, Bruker, Bremen, Germany). In addition, agglutination test was used for confirming *L. pneumophila* (*Thermo Fisher Scientific*, Bred, the Netherlands) and, separately, latex agglutination sera (*Pro-Lab Diagnostics*, Richmond Hill, Canada). The result was expressed as the number of colony forming units for *Legionella* species and strains per one litre of water (CFU/L).

1.6 Culturing of free-living amoebae

The culturing of free-living amoebae (FLA) was performed in accordance with previously described protocols (Vaerewijck et al. 2010). For this purpose, liquid Page`s amoeba Saline (PAS) broth (15 mL) along with two rice grains (*Dobeles dzirnavnieks*, Dobele, Latvia) that were sterilised in a dry air sterilisation oven at +170 ± 2 °C, 2 h were added to a Petri dish containing fragments of membrane filter. The Petri dishes were incubated for 4 to 5 days at 25 ± 2 °C temperature. After incubation, the Petri dishes were inspected under optical microscope at 400× magnification and the genera of amoebae were identified microscopically according to determinants (Smirnov, 1999; Smirnov et Brown, 2004).

1.7 Molecular identification of free-living amoebae species

The amoebae were supplemented before DNA extraction with liquid peptone yeast glucose (PYG) broth (70 µL) (*Biolife Italiana*, Milano, Italy). The DNA extraction was performed with a *Flexi Gene DNA* kit (*QIAGEN*, Hilden, Germany). The amount of DNA was measured with a NanoDrop ND-1000 spectrophotometer (*ThermoFisher Scientific*, Waltham, MA, USA). In the cases

when the concentration exceeded 30 ng/μL, the DNA was diluted with ribonuclease-free water.

Determination of *Acanthamoeba* was carried out with PCR according to a previously described protocol (Schroeder et al., 2001). The following reference materials were used for positive control: *Acanthamoeba quina* ATCC-50241 and *Acanthamoeba castellanii* Neff. ATCC-30010. A replacement of DNA sample with water free of DNA and nucleases was used as a negative control.

The identification of species belonging to *Amoeboidae* and *Vahlkampfiidae* genera was based on a previously described protocol (Le Calvez et al., 2012). The following reference materials were used for positive control: *Vahlkampfia inornata* ATCC-30965 and *Acanthamoeba quina* ATCC-50241. A replacement of DNA sample with water free of DNA and nucleases was used as a negative control.

PCR for determining the representatives of *Vermamoeba* genera (previously *Hartmanella*) was performed according to a previously described protocol (Solgi et al., 2012). A replacement of DNA sample with water free of DNA and nucleases was used as a negative control.

The PCR products were prepared for sequencing after purification, using USB ExoSAP-IT PCR product cleanup reagent (*Affymetrix, Inc.*, Santa Clara, CA, USA). The Big Dye Terminator v3.1 kit (*Thermo Fisher Scientific*, Waltham, MA, USA) was employed according to the manufacturer's instructions. The sequencing of PCR product using both primers was achieved with *Applied Biosystems 3500 Series Genetic Analyzer* (Applied Biosystems, Foster City, CA, USA). The sequencing data were processed with a Mega (*Molecular evolutionary genetics analysis*) program. The homology of the obtained sequences relative to the gene database was analysed with the BLAST program obtained from the US National Biotechnology Information Center (NCBI) website.

1.8 *Legionella* spp. isolates used in the study

A total of 137 *L. pneumophila* cultures from the Microbial culture collection of the Institute of Food Safety, Animal Health and Environment (BIOR)" were used in this study, representing the previously isolated serogroups and possible geographical variations across the territory of Latvia. Out of the total number, 72 isolates were obtained from residential properties in Riga, while 65 isolates came from water supply systems in other cities and towns.

Deep frozen (−80 ± 2 °C) isolates were thawed and seeded on buffered charcoal yeast extract (BCYE) agar growth medium (Oxoid, Basingstoke, United Kingdom) and incubated at 36 ± 2 °C temperature for 48–72 h.

1.9 DNA extraction and complete genome sequencing

DNA extraction was performed after 48 h incubation of *L. pneumophila* at 36 ± 2 °C temperature. Individual colonies were suspended in test tubes with nuclease-free water (500 µL) and then homogenised (*Vortex Genius*, IKA, Staufen, Germany), in order to obtain a homogeneous suspension. Cell lysis was achieved by thermal shock – incubation for 8 minutes at 100 °C temperature. The test tubes were cooled and centrifuged (3 min × 13 000 RPM) and 400 µL portions of the obtained supernatant were transferred to new test tubes. The concentration of DNA was determined with a spectrophotometer (*NanoDrop Technologies*, Waltham, MA, USA).

DNA libraries were prepared using an *Illumina DNA Prep* kit (*Illumina*, San Diego, CA, USA). Sequencing was achieved with *Illumina MiSeq* (*Illumina*, San Diego, CA, USA), using either MiSeq v2 reagent kit with 500 cycles, or v3 kit with 600 cycles (Cat.# MS-102-2003 and MS-102-3003, *Illumina*, San Diego, CA, USA), in order to obtain paired-end reads for each isolate with at least 30 bp overlap.

1.10 Complete genome sequencing data analysis

Low quality basic and sequencing adapters were trimmed from complete genome sequencing data with Trimmomatic v0.38 (Bolger et al., 2014) software. *De novo* assembly of the trimmed reads was performed with SPAdes assembler v3.14.0 (Prjibelski et al., 2020).

Sequence-based typing (SBT) was conducted according to the SBT scheme developed by the ESCMID *Legionella Study Group* (ESGLI) (Gaia et al., 2005; Mentasti et al., 2014). The data were obtained from complete genome sequencing results, taking reconstructed genomes and unprocessed reads with two different data processing tools. Initially, the "legsta" (*In silico Legionella pneumophila Sequence Based Typing*) tool was used (https://github.com/tseemann/legsta/), in order to identify alleles of each SBT locus in the reconstructed genomes. Since one complete genome may contain several different copies of *mompS* gene, a specialized *mompS* tool was used (Gordon et al., 2017). SBT genotypes were visualized as minimum spanning trees with GrapeTree v1.5.0 program (Nascimento et al. 2016). Clonal complexes and singletons were identified with the goeBurst algorithm (Feil et al., 2004; Francisco et al., 2009).

The basic genome multilocus (cgMLST) genotypes were determined in accordance with a *L. pneumophila* cgMLST scheme that was developed before (Moran-Gilad et al., 2015). The cgMLST scheme consisting of 1521 locus was previously elaborated and adapted for allele calling with chewBBACA software v2.8.5 (Silva et al., 2018). Two loci were identified as paralogues, therefore only 1519 loci were taken into account during the cgMLST analysis. CgMLST genotypes were visualized in the form of minimum spanning trees with GrapeTree v1.5.0 (Zhou et al., 2018).

1.11 Identification of virulence and antimicrobial resistance genes

Genes coding for virulence traits were identified in the sequenced *L. pneumophila* isolates obtained from drinking water samples that were collected from residential buildings. A virulence factor database (VFDB) was employed for this purpose (Liu et al., 2022). The database entries were downloaded on November 12th, 2022 and supplemented with DNA sequences of *lvr* and *lvh* loci (GenBank session Y19029.1). Separately, the evaluation of *rtxA* gene was performed in *L. pneumophila* strain AA100 (GenBank ID AF057703.1 nucleobase positions 949-4575). A tool based on BLAST (*abricate* v1.0.1, https://github.com/tseemann/abricate) was used for checking all isolates against this database of virulence-coding genes. Any gene was considered to be present when a BLAST identity of at least 80 % was revealed in at least 80 % of reference sequence length, which is an important quality indicator. In addition, *in silico* PCR analysis with *rtx1/rtxA*-*rtx2/rtxA* and *rtx3/rtxA*-*rtx4/rtxA* primer pairs (Samrakandi et al., 2002) was simulated using the iPCRess tool from Exonerate v2.2.0 software suite (Slater et Birney, 2005). The presence of antimicrobial resistence (AMR) genes was determined using the ResFinder program v4.1.7 (version 2022-05-24) and its associated database (Bortolaia, 2020). The same identity and overlap thresholds were applied to AMR genes as to virulence genes.

1.12 Statistical data processing and analysis

In order to determine the *Legionella* associated seropositivity factors in blood donors, logistic regression analysis was performed. The data were stratified according to the location of residence for each donor. The variables included age, sex, type of hot water supply system, and preceding health episodes. All variables were subjected to univariate analysis, in order to identify

possible risk factors that were included in the multivariate logistic regression model. The statistical analyses were performed using SPSS v.22.0 (SPSS Inc., Chicago, IL, USA) and R version 4.2.3 (2023-03-15 ucrt), 2023 (*The R Foundation for Statistical Computing*, Vienna, Austria).

Contingency tables, $\chi 2$ tests, and ANOVA were used for evaluating the association between *Legionella* spp., free-living amoebae, and other factors including the temperature and extent of colonisation. The maps were composed with QGIS version 3.30.

2 Results

2.1 *L. pneumophila* seroprevalence among blood donors

In general, the level of *L. pneumophila* SG 1–6 seroprevalence among blood donors was 4.8 %. The seroprevalence against the *L. pneumophila* serogroup 1 reached 0.2 % (5 of 2007 blood donors). The sex, age, smoking habits, and the history of Legionnaires' disease, pneumonia, bronchitis or flu-like illness over the previous year were considered as individual factors relevant to this study, which may be associated with seropositivity against *L. pneumophila* (Table 2.1). The seroprevalence among females (5.9 %) was higher than among males (3.3 %). Among the 2007 blood donors, 576 were regular smokers, with 8.2 cigarettes smoked per day on average (ranging from 1 to 30 cigarettes reported in the questionnaire).

Table 2.1

Individual factors associated with *L. pneumophila* SG 1-6 seroprevalence

Age group	Number of samples/Positive samples (%)		
	Total	Females	Males
	2007/96 (4.8)	1121/67 (5.9)	886/29 (3.3)
18–35 years	1109/51 (4.6)	584/33 (5.7)	525/18 (3.4)
36–50 years	581/27 (4.6)	354/21 (5.9)	227/6 (2.6)
51–65 years	317/18 (5.7)	183/13 (7.1)	134/5 (3.7)
Smoking			
Yes	576/18 (3.1)	219/7 (3.2)	357/11 (3.1)
No	1419/78 (5.5)	896/60 (6.7)	523/18 (3.4)
Health history, previous year:			
Bronchitis			
Yes	52/3 (5.8)	38/2 (5.3)	14/1 (7.1)
No	1927/93 (4.8)	1068/65 (6.1)	859/28 (3.3)
Flu-like illness			
Yes	195/19 (9.7)	136/12 (8.8)	59/7(11.9)
No	1781/76 (4.3)	970/54 (5.6)	811/22 (2.7)

The environmental factors considered as potentially linked to seropositivity against *L. pneumophila* were the degree of urbanisation, type of residential building, age of the building, renovation of water-supply system, method of water heating, occupational exposure, and taking showers outside of the residence (Table 2.2). Seroprevalence was higher in larger towns and cities, ranging from 3.5 % in the countryside up to 6.8 % in the capital city Riga. The highest seroprevalence was observed in females living in Riga, in multiapartment buildings with centralised hot water supply (11.2 %; 31 of 277). The lowest seroprevalence was observed in the residents of rural areas lacking access to centralised hot water supply (0.9–2.5 %).

Table 2.2

Environmental factors associated with *L. pneumophila* SG 1–6 seroprevalence

	Number of samples/Positive samples (%)		
	Total	**Females**	**Males**
Urbanisation			
Riga	615/42 (6.8)	358/34 (9.5)	257/8 (3.1)
Other cities, towns	611/27 (4.4)	342/15 (4.4)	269/12 (3.3)
Rural areas	777/27 (3.5)	419/18 (4.3)	358/9 (2.5)
Type of building			
Individual house	666/18 (2.7)	345/10 (2.9)	321/8 (2.5)
Multi-apartment building	1320/77 (5.8)	766/56 (7.3)	554/21 (3.8)
Age of building			
Built before 1950	359/8 (2.2)	196/4 (2.0)	163/4 (2.5)
Built 1951–1970	494/30 (6.1)	286/23 (8.0)	208/7 (3.4)
Built 1971–1990	625/34 (5.4)	340/21 (6.2)	285/13 (4.6)
Built after 1991	187/12 (6.4)	103/9 (8.7)	84/3 (3.6)
Renovation of water-supply system			
Yes	752/31 (4.1)	390/19 (4.9)	362/12 (3.3)
No	936/56 (6.0)	570/42 (7.4)	366/14 (3.8)
Method of water heating			
Centralised/municipal	1027/69 (6.7)	597/51 (8.5)	430/18 (4.2)
Electrical heater	623/16 (2.6)	338/11 (3.3)	285/5 (1.8)
Gas heater	107/5 (4.7)	66/3 (4.5)	41/2 (4.9)
Firewood stove	224/5 (2.2)	112/1 (0.9)	112/4 (3.6)

Table 2.2 continued

Urbanisation	Number of samples/Positive samples (%)		
	Total	Females	Males
Occupational exposure			
Yes	92/2 (2.2)	41/0 (0.0)	51/2 (3.9)
No	1898/93 (4.9)	1073/66 (6.2)	825/27 (3.3)
Showering outside of the place of residence			
Yes	1201/65 (5.4)	654/44 (6.7)	547/21 (3.8)
No	730/26 (3.6)	424/19 (4.5)	306/7 (2.3)

A large proportion (> 60 %) of the study participants confirmed that they had taken shower outside of their place of residence during the previous year. Overall, there was no significant difference between donors who used shower at home only and those who had taken shower also at other locations ($p > 0.05$). Only a small fraction of the donors (4.6 %) were constantly exposed to water aerosols at work, for example, car wash, spa, dental clinics, and factories. Analysis of the data from questionnaires did not reveal substantial differences between the participants with and without occupational exposure or any effects due to wearing protective masks ($p > 0.05$).

The potential risk factors for *L. pneumophila* seroprevalence were evaluated with logistic regression method. The sex of study participants was identified as a risk factor in univariate analysis, as females were more likely to be seropositive than males (OR = 1.88, 95 % CI 1.20–2.93) (Table 2.3).

Table 2.3

The odds ratio (OR) and 95 % confidence interval (CI) for correlation between *Legionella pneumophila* SG 1–6 seropositivity and the anticipated risk factors

Factor	OR	95 % CI
Sex ($p = 0.005$)		
Female vs. male	1.88	1.20–2.93
Type of building ($p = 0.011$)		
Multiapartment vs. individual house	2.23	1.32–3.76

Table 2.3 continued

Factor	OR	95 % CI
Urbanisation (p = 0.037)		
Riga vs. rural areas	2.04	1.24–3.34
Other cities, towns vs. rural areas	1.28	0.75–2.21
Water heating method (p = 0.001)		
Centralised vs. firewood stove	3.16	1.26–7.91
Electrical heater vs. firewood stove	1.16	0.42–3.19
Gas heater vs. firewood stove	2.15	0.61–7.58
Smoking habit (p = 0.027)		
Yes vs. no	0.56	0.33–0.94
Recent fever (p = 0.001)		
Yes vs. no	2.42	1.43–4.10

The type of residential building was another risk factor, with OR = 2.23 and 95 % CI 1.32–3.76 for the inhabitants of multi-apartment buildings, compared to individual family houses. Blood donors from cities and towns, as well as the capital city Riga were seropositive more frequently compared to rural residents (OR = 1.28, 95 % CI 0.75–2.21 and OR = 2.04, 95 % CI 1.24–3.34, respectively). Residents of buildings with centralised hot water plumbing were more frequently seropositive for *L. pneumophila* (OR = 3.16, 95 % CI 1.26–7.91) than the residents of buildings with installed electrical water boilers (OR = 1.16, 95 % CI 0.42–3.19), gas heaters (OR = 2.15, 95 % CI 0.61–7.58) or wood-fired stoves. Previous medical history of fever was identified as a risk factor (OR = 2.42, 95 % CI 1.43–4.1), while other medical episodes, including pneumonia and bronchitis, were not associated with *L. pneumophila* seropositivity. Miscellaneous anticipated risk factors that were not found to be associated with seropositivity were the age of blood donors, the age of their housing, renovation status of residential water supply systems, shower use outside of the place of residence, and occupational exposure to water mists. The risk factors identified in univariate analysis were included in the multivariate logistic regression model. The type of water heating system in residential

buildings, the sex of study participants, and medical history of fever episodes were identified as the main risk factors for *L. pneumophila* seropositivity.

2.2 The occurrence of *Legionella* spp. in drinking water supply systems and the factors affecting its persistence and colonisation ability

The presence of *Legionella* spp. was found in a total of 490 water samples out of 1467 (Table 2.4). At least one *Legionella* spp. positive sample was discovered in 176 of 317 buildings (55.5 %).

Table 2.4

The total number of samples and the *Legionella* spp. positive samples

Type of building	Total number		*Legionella* spp. positive	
	Buildings	Samples	Buildings (positive %)	Samples (positive %)
Residential buildings	210	521	118 (56.2)	207 (39.7)
Riga	140	338	80 (57.2)	135 (39.9)
Other cities and towns	70	183	38 (54.3)	72 (39.3)
Hotels	81	903	48 (59.3)	266 (29.4)
Riga	55	739	29 (52.7)	189 (25.6)
Other cities and towns	26	164	19 (73.1)	77 (47.0)
Other buildings	26	43	10 (38.5)	17 (39.5)
Riga	9	19	5 (55.6)	11 (57.9)
Other cities and towns	17	24	5 (29.4)	6 (25.0)
Total	317	1467	176 (55.5)	490 (33.4)

The most often isolated *Legionella* species was *L. pneumophila*, which was found in 482 of 490 *Legionella* spp. positive samples (98.4 %). *L. rubrilucens* was identified in nine samples (1.8 %), and *L. anisa* was present in two samples (0.4 %). Simultaneous contamination with two *Legionella* species was discovered in three cases. Thus, two different buildings each had

one sample showing the presence of both *L. pneumophila* and *L. rubrilucens*. In another building, there was a simultaneous presence of *L. pneumophila* and *L. anisa.*

The presence of *Legionella* in hot water was found more often ($p < 0.05$) than in cold water (Table 2.5). Water samples taken outside of Riga showed more frequent presence of *Legionella*. This trend was not statistically significant for residential buildings, while hotels outside of Riga had a substantially higher occurrence of *Legionella* ($p < 0.0001$).

Table 2.5

The occurrence of *Legionella* spp. in cold and hot water

Type of building	Cold water		Hot water	
	Number of samples	Positive (%)	Number of samples	Positive (%)
Residential buildings	**176**	**44 (25)**	**345**	**163 (47.2)**
Riga	99	28 (28.3)	239	107 (44.8)
Other cities and towns	77	16 (20.8)	106	56 (52.8)
Hotels	**–**	**–**	**903**	**266 (29.5)**
Riga	–	–	739	189 (25.6)
Other cities and towns	–	–	164	77 (47)
Other buildings	**16**	**4 (25)**	**27**	**13 (48.1)**
Riga	8	4 (50)	11	7 (63.6)
Other cities and towns	8	0	16	6 (37.5)
Total	**192**	**48 (25.0)**	**1275**	**442 (34.7)**

Serotyping of all isolated *L. pneumophila* samples allowed to identify six serogroups. The predominant *L. pneumophila* serogroup was SG 3, which was found in 208 of 482 cases (43.2 %). *L. pneumophila* SG 2 was found in 176 cases (36.5 %), SG 1 was present in 66 isolates (13.7 %), while serogroups 6 and 9 were identified in 15 and 16 cases, respectively. Only one *L. pneumophila* sample belonged to serogroup 8. In seven cases, *L. pneumophila* belonging to

two different serogroups were simultaneously present in the same water samples. In all of these cases, one of the serogroups was SG 3, while the other serogroup was SG 2 in five cases or SG 9 and SG 1 in one case each.

The observed level of colonisation by *L. pneumophila* varied from 50 CFU/1L, which is the quantification limit of the method, to 1.7×10^4 CFU/1L, with the mean value of 1.8×10^3 CFU/1L. The colonisation of hot water supply systems in residential buildings was higher than in hotels ($p < 0.001$), furthermore, the highest contamination levels were found in residential buildings located in the capital city Riga. On the other hand, the highest concentrations of *Legionella* among hotel water supply systems were discovered in regional cities and towns ($p < 0.01$). The concentration of *Legionella* exceeded 1000 CFU/1L in 46.3 % of cases. This concentration was exceeded in 50.3 % of residential buildings and 32.1 % of hotels (Table 2.6).

Table 2.6

The presence of *Legionella* spp. in water samples

Type of building	Hot water		Cold water	
	Mean colonisation level, CFU/1L (max)	Exceeding 1000 CFU/1L %	Mean colonisation level, CFU/1L (max)	Exceeding 1000 CFU/1L %
Residential buildings	**$2.1 \cdot 10^3$ ($1.3 \cdot 10^4$)**	**50.3**	**$1.7 \cdot 10^3$ ($1.7 \cdot 10^4$)**	**31.2**
Riga	$1.8 \cdot 10^3$ ($1.3 \cdot 10^4$)	54.8	$1.6 \cdot 10^3$ ($1.1 \cdot 10^4$)	28.6
Other cities and towns	$2.6 \cdot 10^3$ ($1.3 \cdot 10^4$)	42.4	$1.9 \cdot 10^3$ ($1.7 \cdot 10^4$)	35.0
Hotels	**$1.2 \cdot 10^3$ ($1.1 \cdot 10^4$)**	**32.1**	**–**	**–**
Riga	$1.0 \cdot 10^3$ ($1.1 \cdot 10^4$)	28.4	–	–
Other cities and towns	$1.6 \cdot 10^3$ ($1.1 \cdot 10^4$)	50.0	–	–

The analysis of *L. pneumophila* colonisation levels in hotels (Table 2.7) revealed a statistically insignificant reduction of *L. pneumophila* colonisation after running the water flow for 3 min ($p = 0.16$). However, the data also showed that in 53 % of hotels where the level of *L. pneumophila* exceeded 1000 CFU/1L (4.4×10^3 CFU/1L on average), running the water stream reduced the colonisation level below 1000 CFU/1L (3.0×10^2 CFU/1L on average) at the point of water use.

Table 2.7

The levels of *L. pneumophila* colonisation before and after running the water flow

Colonisation level, CFU/1L	Before running water stream	After running water stream
Min	50	50
Max	$1.1 \cdot 10^4$	$9.0 \cdot 10^3$
Average	$1.7 \cdot 10^3 \pm 2.8 \cdot 10^2$	$1.2 \cdot 10^3 \pm 1.8 \cdot 10^2$

2.2.1 Water temperature

The average circulating water temperature in the hot water supply systems was 47.8 ± 0.7 °C. Temperature measurements indicated that only in 249 of 1275 hot water sampling occasions (19.5 %) the temperature exceeded 55 °C. No substantial differences between regions, building types, or sampling seasons were found, however, it is important to note that the average temperature of hot water was lower in those cases when the presence of *Legionella* was found ($p < 0.0001$) (Table 2.8).

Table 2.8

The average temperature in hot water supply systems

Type of building	Circulating water temperature		*Legionella* negative samples		*Legionella* positive samples	
	Mean hot water t° °C	Mean cold water t° °C	Mean hot water t° °C	Mean cold water t° °C	Mean hot water t° °C	Mean cold water t° °C
Residential buildings	**51.0**	**13.4**	**51.3**	**12.9**	**50.6**	**14.7**
Riga	49.9	13.7	50.1	13.1	49.7	15.4
Other cities and towns	52.9	12.9	54.2	12.8	51.9	13.7
Hotels	**46.9**	**12.4**	**48.0**	**12.4**	**44.4**	**–**
Riga	48.3	12.4	49.0	12.4	46.0	–
Other cities and towns	42.1	–	42.8	–	41.4	–
Other buildings	**38.2**	**15.3**	**40.8**	**12.9**	**30.5**	**20.0**
Riga	51.0	18.9	51.0	15.5	–	20.0
Other cities and towns	34.0	12.4	35.8	12.4	30.5	–
Total	**47.8**	**13.5**	**48.5**	**12.9**	**46.4**	**15.1**

The temperature of hot water in hotels was measured before and after allowing the water to flow for 3 min (Table 2.9). The analysis of the obtained data showed that the hot water temperature did not reach 50 °C after flowing for 3 min in 35 % of the hotels, while in 27 % of the hotels this temperature was in the range from 50 °C to 55 °C and in 38 % of the hotels the hot water temperature at the point of use exceeded 55 °C. The temperature of hot water increased by 8.4–39.0 °C during the first three minutes of flowing, with the average increase of 23.8 ± 1.2 °C.

Table 2.9

The temperature of hot water in hotels initially and after flowing for 3 min

Temperature, °C	Initially	After flowing for 3 min
Min	16.2	27.7
Max	62.9	68.8
Mean	35.7 ± 0.7	49.8 ± 0.4
Mod	27.0	47.0

The average water temperature in hotels was not significantly different between Riga and other cities or towns both initially ($p = 0.97$) and after flowing for 3 min ($p = 0.66$). Even though the water temperature did not exceed 55 °C in the majority of hotels, it was observed that allowing the water to flow could significantly increase the water temperature ($p < 0.0001$). Data analysis did not reveal any substantial relationship between hot water temperature and the level of colonisation by *L. pneumophila*.

The temperature of hot water was higher in residential buildings with previous cases of Legionnaires' disease ($p < 0.0001$) (Table 2.10).

Table 2.10

The prevalence of *Legionella* spp. in residential buildings with and without known cases of Legionnaires' disease

	Buildings with known cases of Legionnaires' disease		**Buildings without known cases of Legionnaires' disease**		**Total**	
	Number of samples/ positive (%)	**The average water temperature t° °C**	**Number of samples/ positive (%)**	**The average water temperature t° °C**	**Number of samples/ positive (%)**	**The average water temperature t° °C**
Cold water	120/22 (18.3 %)	12.9 ± 0.4	44/19 (43.2 %)	15.6 ± 0.7	164/41 (25.0 %)	13.5 ± 0.3
Hot water	242/107 (44.2 %)	52.1 ± 0.4	86/49 (57.0 %)	45.8 ± 1.1	328/156 (47.6 %)	50.7 ± 0.4

The prevalence of *Legionella* spp. in cold water from buildings with known cases of Legionnaires' disease was substantially lower ($p < 0.0001$), while in buildings without known cases of Legionnaires' disease there was no substantial difference between the prevalence of *Legionella* spp. in cold and hot water ($p = 0.192$). The overall prevalence of *Legionella* spp. was higher in both cold and hot water samples ($p < 0.01$) from multi-apartment buildings without

known cases of Legionnaires' disease, even though the difference for hot water was not statistically significant (p = 0.056).

2.2.2 The occurrence of free-living amoebae in drinking water supply systems

The presence of free-living amoebae was determined in 268 samples, of which 101 were collected from the central and left bank of Daugava districts in Riga, which receive treated surface water, while 167 samples were collected from the right bank of Daugava districts in Riga, as well as other regions of Latvia were subsurface aquifers are used for water supply.

Free-living amoebae were detected in 207 of 268 water samples (Table 2.11), including 37 cold water samples (84.1 %) and 170 hot water samples (75.9 %). At least one detection of free-living amoebae was made in 83 out of 92 buildings (90.2%).

Table 2.11

The total number of samples and the detections of free-living amoebae

	Water source/ number of samples (positive; %)				
	Treated surface water		**Subsurface aquifers**		**Total**
	Cold water	**Hot water**	**Cold water**	**Hot water**	
Multi-apartment buildings	4 (2; 50.0 %)	13 (6; 46.1 %)	24 (21; 87.5 %)	41 (31; 75.6 %)	82 (60; 73.2 %)
Hotels	0 (0; 0.0 %)	75 (55; 73.3 %)	0 (0; 0.0 %)	68 (54; 79.4 %)	143 (109; 76.2 %)
Public buildings	4 (4; 100.0 %)	5 (3; 60.0 %)	12 (10; 83.3 %)	22 (21; 95.4 %)	43 (38; 88.4 %)
Intermediate result	8 (6; 75.0 %)	93 (64; 68.8 %)	36 (31; 86.1 %)	131 (106; 80.9 %)	268 (207; 77.2 %)
Total	101 (70; 69.3 %)		167 (137; 82.0 %)		

Chi squared tests did not indicate a correlation between the type of water (i.e., cold or hot) and the presence of free-living amoebae (χ^2= 1.4; p = 0.33). At the same time, greater diversity of free-living amoebae was observed in hot water samples (χ^2 = 10.3; p = 0.035). The presence of free-living amoebae was considerably higher in those hot water samples with temperature below 50 °C (χ^2 = 21.3; p < 0.0001), while the temperature of cold water had no effect on the presence of free-living amoebae.

The season of the year and the type of building had no impact on the presence of free-living amoebae (χ^2 = 6.0; p = 0.11 and χ^2 = 3.9; p = 0.14, respectively). The occurrence of free-living amoebae was higher in samples from buildings that were supplied with water from subsurface aquifers (χ^2 = 5.8; p = 0.024), while the source of water had no effect on the diversity of amoebae (χ^2 = 7.5; p = 0.11).

A total of eight free-living amoebae genera were detected in 207 samples. At least one half of the samples contained *Acanthamoeba* spp., while five other genera (*Flamella* spp., *Centropyxis* spp., *Vrihiamoeba* spp., *Echinamoeba* spp., and *Tetramitus* spp.) were found in a total of 3 % of the samples.

Only one genus of free-living amoebae was observed in 47.4 % of the samples (127 of 268 samples). Two different genera were found in 69 samples (25.7 %), while 10 samples (3.7 %) contained 3 genera, and one sample (0.4 %) – four different species of amoebae.

Molecular identification of free-living amoebae to the level of species was performed for 81 samples. A total of 13 free-living amoebae species were identified: *Acanthamoeba triangularis*, *A. polyphaga*, *A. castellanii*, *A. haelayi*, *A. quina*, *A. lugdunensis*, *Vermamoeba vermiformis*, *Naegleria neopolaris*, *N. Fowleri*, *Echinamoeba exudans*, *Tetramitus dokdoensis*, *Vrihiamoeba italica*, and *Flamella arnhemensis.* The most common among the identified species were *V. vermiformis* (51.9 %), *A. castellanii* (13.6 %), and *A. polyphaga* (8.6 %). The

representative of *Centropyxis* genus was not identified, although it was present in two cold water samples along with *A. triangularis*.

The occurrence of *Legionella* spp. was considerably higher in samples with less diversity of free-living amoebae ($\chi^2 = 64.9$; $p < 0.0001$). Most frequently, *Legionella* spp. was detected in samples where only one genus of free-living amoebae was present.

The diversity of free-living amoebae had no effect on the *Legionella* species ($\chi^2 = 1.9$; $p = 0.758$), serogroups ($\chi^2 = 4.6$; $p = 0.797$), or the number of colony forming units ($\chi^2 = 7.9$; $p = 0.247$). Only two genera of amoebae showed a substantial association with the presence of *Legionella* spp. – *Acanthamoeba* spp., observed in 146 samples ($\chi^2 = 19.7$; $p < 0.0001$) and *Vermamoeba* spp., observed in 77 samples ($\chi^2 = 7.8$; $p = 0.006$), while other genera had no statistically significant correlation with *Legionella* spp. There was a clear link between the presence of free-living amoebae and the contamination with *Legionella* spp. ($\chi^2 = 58.5$; $p < 0.0001$). No *Legionella* spp. positive samples were found where amoebae were absent and the coexistence of *Legionella* spp. with free-living amoebae reached 55.1 %.

2.3 The results of *Legionella pneumophila* sequence-based and cgMLST typing

Information was obtained about the sequence types of all 137 isolates through complete genome sequencing and data analysis regarding seven alleles *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA* or *neuAh*. Among the 137 sequenced *L. pneumophila* isolates, 72 were from Riga (Annex 3) and 65 from other regions of Latvia (Annex 4).

The 137 strains of *L. pneumophila* included in the study were characterised by 46 sequence types, of which 10 sequence types are new, previously unreported, and lacking a sequence number in the international ESGLI database.

The most common sequence types were ST-338 (18 isolates), ST-366 (16 isolates), and ST-1104 (15 isolates). Among the 46 sequence types identified during this study, 39 (78 %) were observed in only one *L. pneumophila* serogroup each, but there were several sequence types, the isolates of which belonged to more than one serogroup: ST-338 and ST-336 included isolates belonging to three serogroups, while ST-9, ST-787, ST-1354, ST-1362, and ST-1987 *L. pneumophila* strains belonged to two different serogroups (Figure 2.1). Four sequence types (ST-2579-J, ST-2580-J, ST-2581-J, and ST-2582-J) were already previously registered in the EWGLI database as new sequence types of environmental isolates from Latvia.

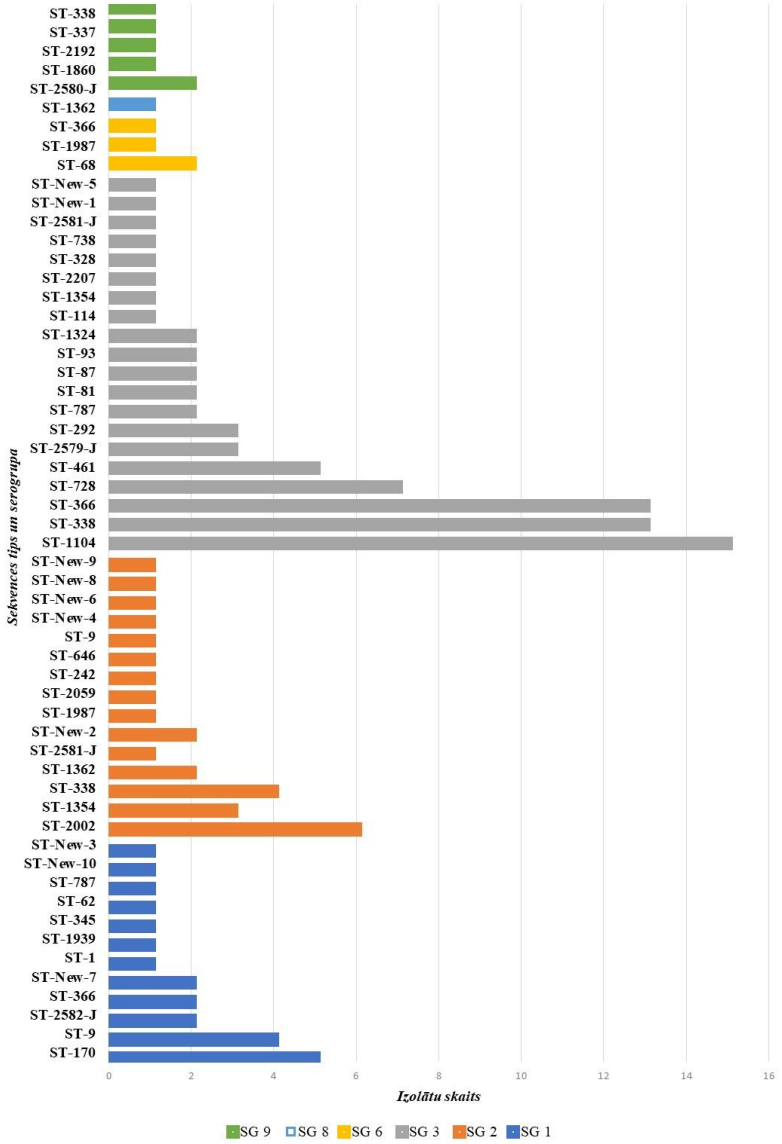

Figure 2.1 ***Legionella pneumophila*** **sequence types**

A total of 46 sequence types from 137 isolates are presented.

A minimum spanning tree graph was constructed for 46 sequence types using the GrapeTree v.1.5.0 and goeBurst algorithms. In the cases of at least 5 matching alleles, seven clonal complexes were identified and circled (Figure 2.2). Two clonal complexes were formed within one serogroup (SG 1), while the isolates grouped in other complexes belonged to different serogroups. Only the SG 8 isolate was not part of any clonal complex.

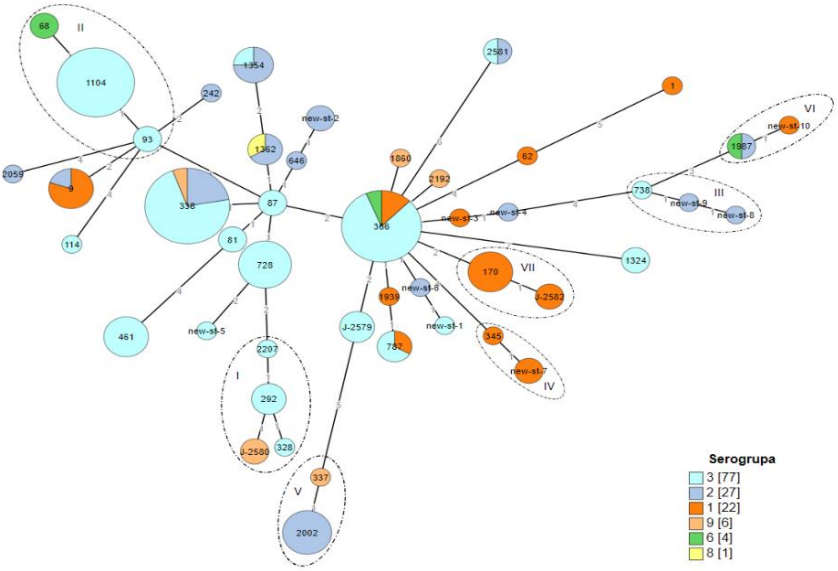

Figure 2.2 **A minimum spanning tree visualization of 46 *L. pneumophila* sequence types**

The colours of individual nodes denote specific serogroups. The seven identified clonal complexes are marked with Roman numerals.

A total of 116 genotypes were obtained by cgMLST typing (Figure 2.3). The isolates typed by either SBT or cgMLST methods did not show substantial geographical trends. No sequence types were discovered in this study that would be unique to certain towns or regions. The sequence types found at one location

were found also in other cities or towns, and the *L. pneumophila* strains found in a specific city could belong to different clonal complexes. For example, two different buildings in the town of Madona yielded two sequence types that belonged to clonal complexes I and VII. All cities and towns except Riga provided no more than seven *L. pneumophila* isolates that were found in no more than two buildings. For example, ST-461 was found only in the town of Talsi, five different samples from a single building. While in the SBT minimum spanning tree it appeared as one node, in the case of cgMLST those were 4 separate, slightly different cgMLST types where two isolates were recorded as identical and three isolates as different from both the two identical ones, as well as from each other.

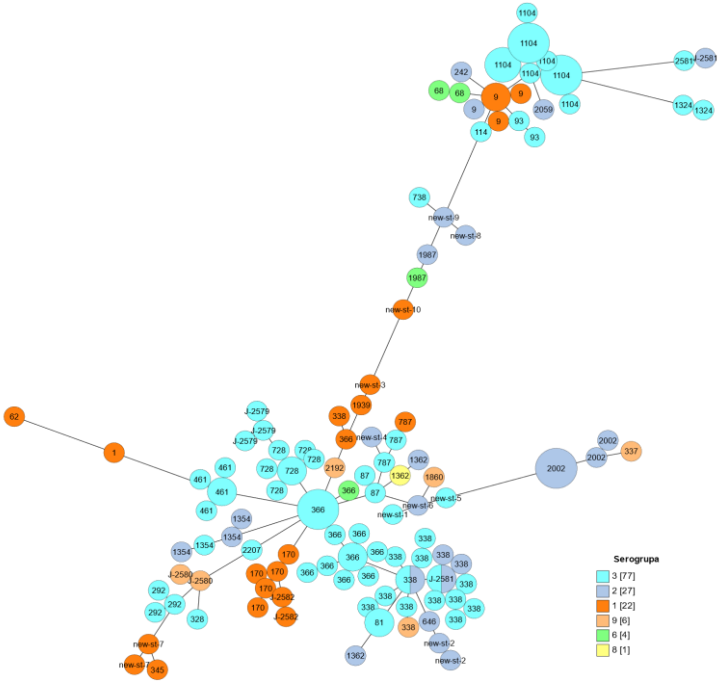

Figure 2.3 **A minimum spanning tree visualization of 116 *L. pneumophila* cgMLST types**

The colours of individual nodes denote specific serogroups.

2.4 Genes coding for virulence factors and antimicrobial resistance

The characterization of genes coding for virulence factors was performed for all 58 strains of *L. pneumophila* that were isolated from water samples obtained in residential buildings. A total of 420 virulence genes representing 59 gene families were found in the 58 sequenced *L. pneumophila* genomes. The number of genes in one isolate ranged from 312 to 415, with each isolate containing 375 virulence genes on average. Similar genetic diversity was

obtained among isolates from buildings associated with LD cases and from buildings without known LD cases.

Only one antimicrobial resistance gene was found among all sequenced *L. pneumophila* isolates, namely, *aph(9)-la*, which codes for the antimicrobial resistance factor spectinomycin phosphotransferase.

Each of the 58 isolates contained 260 (62.1 %) virulence genes. Each virulence gene was found in 52 isolates on average. For this study, 11 genes with substantial role in the infectiousness of *L. pneumophila* towards humans were selected: *enhC*, *htpB*, *omp28*, *mip*, *mavC*, *legK1*, *sidj*, *lvhD4*, *lpnE*, *lspC*, and *rtxA*.

The genes *enhC*, *htpB*, *omp28*, *mip*, *lpnE* and 11 genes from the *lsp* gene family were identified in all 58 *L. pneumophila* isolates.

No substantial differences were found in the relative occurrence of genes between buildings with cases of Legionnaires' disease and buildings without such cases ($p > 0.05$), and between different serogroups, except *sidJ*, which was less frequent in SG 9 isolates than in SG 1, SG 2, and SG 3 isolates ($p < 0.05$), and the PCR simulated *rtxA*, which was less frequent in SG 1 isolates compared to the SG 3 isolates ($p < 0.05$).

Overall, 260 genes were observed among all isolates (62.1 %), including *enhC*, *htpB*, *omp28*, *mip*, *lpnE* (Figure 2.4), and 11 genes of the lsp family. The *Core Genome SNP* graph shows the same groups as the cgMLST minimum spanning tree, and the separate virulence genes of the *leg* family, which were observed in only 7 isolates of 58, were part of a group formed around ST1104.

The *Mav* family was represented by 13 identified genes, of which nine were found in all 58 isolates, while *mavC* was found in 54, but *mavG* 53, *mavH* 55, and *mavI* were found in 57 isolates. A total of 29 virulence genes belonging to the *leg* family were identified. The occurrence rate of *Leg* genes among *L. pneumophila* isolates varied from 12 % to 100 %, but fewer than 50 isolates

of 58 contained only 8 virulence genes of the *leg* family. Among the 11 virulence genes representing the *sid* family, all 58 isolates revealed the presence of *sidA*, *sideE*, *sidF*, and *sidK*. On the other hand, *SidG* and *sidH* were the least common and were found in 6 and 13 isolates, respectively. All 11 identified genes of the *lvh* family were found in 46 isolates, except for *lvhB2*, which was found only in 27 isolates from residential buildings.

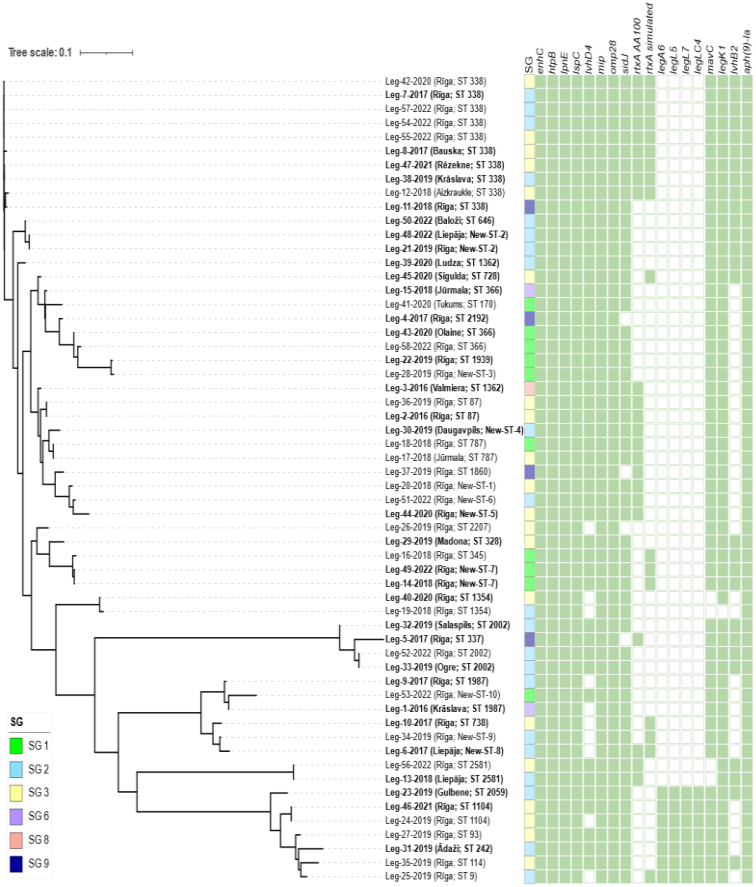

Figure 2.4 **The maximum-likelihood tree formed by comparison |of basic genome from 58 *L. pneumophila* strains**

The city or town of sample origin and the SBT type are given in parentheses after each name, while isolates from buildings with a history of Legionnaires' disease cases are emphasized in bold. Coloured squares indicate the serogroup of each isolate. The panel of green squares shows the distribution of *L. pneumophila* virulence genes and antimicrobial resistance genes among all isolates. The green filled squares point to the presence of a gene, while empty squares indicate the absence of gene in the particular *L. pneumophila* isolate.

3 Discussion

3.1 The seroprevalence of *L. pneumophila* and the associated factors

Regular and prolonged exposure to water aerosols contaminated with *Legionella* spp. elicit immune response and antibody formation in humans, which can last at measurable levels for several months and even years, while not causing any symptoms. The results obtained in this study showed that the average seroprevalence in Latvia against *L. pneumophila* serogroups 1–6 was 4.6 %. Furthermore, this seroprevalence depended on the extent of urbanisation and varied from 3.5 % on average in the countryside to 9.5 % for the residents of multi-apartment buildings in Riga.

The main reservoirs for *Legionella* are artificial water environments and there are multiple reports about *Legionella* contamination in residential water plumbing as a major problem (Dilger et al., 2018; Felice et al., 2019; Gleason et al., 2023). Furthermore, as demonstrated by earlier seroprevalence studies, antibodies against *Legionella* are more common among city dwellers, additionally confirming that *Legionella* contamination is endemic in cities (Graham et al., 2020). Earlier studies from Denmark revealed a seroprevalence level of 22.9 % among blood donors (Rudbeck et al., 2008), while among healthy individuals in Sweden the seroprevalence was around 1.0 % (Darelid et al., 2003). Looking at the Southern Europe, the seroprevalence level in Italy against *L. pneumophila* SG 1–6 was 3.4 % and 16.4 % against *L. pneumophila* SG 7-14 (Borella et al., 2008), while a study from France concerned about exposed or non-exposed industrial workers showed a low prevalence of 2.8 % (Daniau et Cabanes, 2010). The major factors responsible for the observed differences in *L. pneumophila* seroprevalence levels are the non-standardised testing methods and variations in study designs.

Substantial ($p = 0.005$) divergence was observed in *L. pneumophila* seroprevalence among females (5.9 %) and males (3.3 %), which was not previously recognized as a risk factor for Legionnaires' disease (Den Boer et al., 2006). The incidence of Legionnaires' disease has been reported to be higher among men (ECDC, 2022). A report from Japan also indicated a substantially higher fraction of men vs. women among the patients infected with *L. pneumophila* SG 1, compared to patients infected by other *L. pneumophila* serogroups (Amemura-Maekawa et al., 2010). The elevated seroprevalence among females in Latvia can be probably associated with greater exposure to water aerosols during household chores. Women may be frequently exposed to small doses of *Legionella*, unable to cause illness but eliciting immune response (Rozentale et al., 2011), but there is also a more likely hypothesis about genetic reasons for this sexual dimorphism of infectious diseases (Gay et al., 2021). Several major regulatory and metabolic proteins involved in signal transduction, apoptosis, and carbohydrate metabolism are coded in the X chromosome, and their polymorphic variants are widespread in the human population (Spolarics et al., 2017). Women may be more resistant against the Legionnaires' disease due to polymorphism of Toll-like receptors (TLR), which may be instrumental in the resistance against *Legionella* and in other immunogenetic factors (Hawn et al., 2005).

A remarkable association between the smoking habits and seropositivity was observed during this study. According to the study results, seroprevalence was considerably lower among women who smoke, compared to non-smokers. Only 3.2 % of female blood donors who smoke were seropositive for *L. pneumophila*, while the seropositivity rate among non-smokers reached 6.7 % (OR = 0.46). This trend may be linked to the inhibitory effect of smoking on the protective functions of humoral immunity. As reported by other researchers, smoking of tobacco was associated with a lower IgG level (Feldman et al., 2013),

and the reason may be linked to the aforementioned polymorphism of Toll-like receptors. TLR2 plays an important role in the recognition of *L. pneumophila* and it is known that TLR6 and TLR1 interact with TLR2 during early recognition of the infection. A research group from the Netherlands investigated the polymorphism of TLR1, TLR2, and TLR6 in 98 Legionnaires' disease patients and 268 persons in the control group (Misch et al., 2013). No link was found between the TLR1 and TLR2 polymorphism and Legionnaires' disease cases, but TLR6 polymorphism 359T > C (rs5743808) was associated with elevated risk of Legionnaires' disease (OR = 5.83) and this risk was substantially higher among smokers. The authors of the study asserted that TLR6 polymorphism 359T > C may also serve as a separate tool for the identification of genetically determined increased risk of Legionnaires' disease.

An additional explanation for our observed inverse result among female smokers may be the limitations of study population, because blood donors represent the healthier part of society, and this study was not focussed on the recruitment of smokers.

Only a small fraction of blood donors in our study were continuously exposed to water aerosols at the workplace (4.6 %), while one study participant reported the use of personal protective equipment. Studies have shown that a range of professions associated with water aerosols and cooling systems may carry an increased risk of *Legionella* infections (Principe et al., 2017), emphasizing healthcare specialists and in particular the field of dentistry as one of the affected job categories, but the results remain somewhat inconclusive. An earlier meta-analysis failed to provide scientific proof that dentistry would carry an increased professional risk (Petti and Vitali, 2017). However, as recognized by the researchers, there may be substantial differences between different plumbing systems and the implementation of infection control guidelines, which may strongly influence the risks.

The collected data did not reveal blood donors with Legionnaires' disease in their health history. Also, no substantial differences were observed between blood donors with or without a history of pneumonia or bronchitis during the preceding year. However, substantial differences were found between blood donors of both sexes and all ages who suffered from a flu-like illness during the previous year (OR = 2.42, p = 0.001), possibly pointing to undiagnosed cases of Pontiac fever that were missed by the healthcare system (Hamilton et al., 2018).

The type of residential building, degree of urbanisation, and the method of hot water supply were the most significant environmental factors identified during our study. The inhabitants of multi-apartment buildings were subjected to a greater risk than the residents of individual houses (OR = 2.23, p = 0.011). Furthermore, geographical areas with higher population density were linked to a larger probability of seropositive status (OR = 1.89, p = 0.046). Also, higher risk of seropositivity was in buildings with centralised hot water plumbing where the residents could control neither the hot water temperature, nor the overall condition of the plumbing (OR = 3.16, p = 0.001). Thus, the type of hot water supply had the strongest effect as revealed by multifactorial logistic regression analysis. These findings are in line with previous environmental studies where *Legionella* contamination was more common in buildings with centralised hot water plumbing (Kruse et al., 2016). More than 67 % of the study participants live in buildings built between 1950's and 1990's and the plumbing systems had not been renovated in the majority of cases. However, previous research has confirmed equal probability of *Legionella* contamination in old and new buildings, while direct disinfection measures can improve public health (Donohue et al., 2022).

The main factor associated with *L. pneumophila* seropositivity according to this study was centralised hot water plumbing in residential buildings. Proper servicing of such hot water systems can be recognized as essential to public

health. Building managers must ensure correct maintenance, disinfection, and appropriate circulating water temperature settings. Effective strategies for *Legionella* prophylaxis must include both the education of residents and training of facility managers, introduction of risk-based monitoring programmes, and careful selection of laboratory testing methods for the investigation of environmental safety and clinical cases.

3.2 The occurrence of *Legionella* spp. in water supply systems and factors affecting its persistence and colonisation

Legionnaires' disease is a preventable infection with increasing incidence, and centralised hot water supply systems in buildings are a significant source of sporadic cases of this disease. The results of this study showed that *Legionella* are widespread in Latvia. The water supply systems of multi-apartment buildings, hotels, gyms, and office buildings may serve as potential sources of *Legionella* infections.

The occurrence and diversity of *Legionella* in 210 multi-apartment buildings, 81 hotels, and 26 public buildings throughout Latvia were determined during this study. Overall, *Legionella* were found in 55.6 % of buildings and 33.4 % of water samples, including 56.2 % of residential buildings and 39.7 % of water samples from households. These results considerably exceed the occurrence in residential buildings reported from other countries – 20.7 % (Dilger et al., 2018) and 32.7 % (Kruse et al., 2016) in Germany, as well as two reports from Italy where the prevalence *Legionella* in hot water supply systems varied from 19.8 % (Felice et al., 2019) to 26 % (Totaro et al., 2017). In the USA, at least one *Legionella* positive water sample was found in 15 % of single-family houses (Gleason et al., 2023). Similar studies from Japan revealed the presence of *Legionella* upon culturing in 6.5 % of water samples (Kuroki et al.,

2017), while in the UK the presence of *Legionella* was detected in 8 % of water samples from showers in households (Collins et al., 2017).

Earlier studies about the occurrence of *Legionella* in the water supply systems of hotels found similar prevalence: 20.7 % of samples from 62.95 % of hotels in Greece contained *Legionella* spp. (Papadakis et al., 2021), compared to 25.6 % of water samples from 57.15 % of hotels in Italy (De Filippis et al., 2017), 15.9 % of hot water samples from 65.4 % of locations in the Balearic islands of Spain (Domenech-Sanchez, Laso, Berrocal et Alberti, 2022), and 17 % of samples from 60 % of selected hotels in Israel (Yakunin et al., 2020). *Legionella* were also found in 25.7 % of samples from hotels in Bosnia and Herzegovina (Bešič et al., 2021). The lowest occurrence of *Legionella* was observed in the Canary Islands – only 8.5 % of samples from hot water systems were contaminated (Domenech-Sanchez, Laso et Alberti, 2022). Overall, except for the study by Domenech-Sanchez (Domenech-Sanchez, Laso et Alberti, 2022), quite similar levels of contamination by *Legionella* were observed. The prevalence of *Legionella* may be different in specific countries or studies, yet it is difficult to compare data obtained according to various sampling plans that included large-scale monitoring programmes, more conveniently focussed studies, and targeted investigations in response to clusters of Legionnaires' disease cases. Different countries may have non-standardised monitoring and control requirements and regulations of the minimum hot water temperature.

It should be noted that the fraction of positive samples was substantially higher at hotels outside of Riga (47 %), while only 25.6 % of samples from hotels in Riga were positive. The surface waters used for drinking water supply of Riga are treated, while subsurface water used in the regions are not additionally purified. Thus, it can be asserted that the municipal water supply of Riga has been thoroughly disinfected. However, it can be further argued that the main reason for lower prevalence of *Legionella* in Riga hotels may be superior

management practices and higher quality standards, since the majority of hotels in the capital city are managed by international hotel chains. In addition, regional hotels are typically smaller and less often booked, resulting in frequent stagnation of water, promoting the propagation and persistence of *Legionella*.

Contamination by *Legionella* was 13 % more common in water samples from buildings not previously associated with cases of Legionnaires' disease. The average temperature of hot water in such buildings was lower by seven degrees (mean value 45.8 °C) than in buildings with previous cases of Legionnaires' disease (mean value 52.1 °C). The circulating hot water temperature or temperature at the points of use are not currently regulated in Latvia. Building managers are only obliged to ensure hot water temperature at the exit from heat exchanger, which must be at least 55 °C according to the Cabinet of Ministers regulations No. 906. Taking into account the considerable length of plumbing and heat loss between the hot water heat exchanger and shower heads, the observed mean temperature of 45.8 °C at the points of use is to be expected in buildings without a history of Legionnaires' disease. On the other hand, as a consequence of Legionnaires' disease cases, the building managers are required to perform disinfection procedures, monitoring of *Legionella*, and take preventive measures, although the frequency and guidelines have not been set. Therefore, the managers of buildings with previous cases of Legionnaires' disease are likely to take extra measures and provide a higher hot water temperature as a precaution, resulting in a lower incidence of culturable *Legionella*.

The frequent occurrence of *Legionella* in the water supply in Latvia is likely linked to ineffective maintenance of plumbing, because water temperature is one of the main factors facilitating the persistence and propagation of *Legionella* in the water supply systems of buildings (Singh 2022; Kruse, 2016). In our study, the average hot water temperature was 47.8 °C, which is suitable

for the viability of *Legionella*, and only in 19.5 % of cases the hot water temperature exceeded 55 °C. The temperature of 55 °C has been previously identified as borderline for effectively reducing the colonisation by *Legionella* (Rasheduzzaman et al., 2020), and would be the recommended minimum hot water temperature at the point of use, for avoiding the propagation of *Legionella* (Lévesque et al., 2004; ESGLI, 2017). The inadequate temperature in hot water supply systems can be attributed to several causes. First, it reflects the overall situation in the economy and the intent to conserve energy. Second, it may be linked to insufficient training of the personnel, especially in public buildings and hotels. Third, in many older buildings that have not seen renovation of water plumbing, there may be technical limitations to raising the temperature, if the heritage systems are not capable of maintaining water temperature at least at the level of 55 °C.

Close parallels were observed between this study and another study from Hungary, where 1809 water samples were collected from 168 different buildings from year 2006 until 2013, and 60 % of the buildings were colonised by *Legionella*, while 46 % of hot water samples were positive for *Legionella* (Barna et al., 2016). The main causes for the high contamination level were deemed to be similar in both countries. The Hungarian research team also pointed to the inadequate water temperature and the lack of sufficient control and risk management efforts.

In this study, six *L. pneumophila* serogroups were identified, among which the dominant ones were SG 3 (43.2 %), SG 2 (36.5 %), and SG 1 (13.7 %). These results are in agreement with the low occurrence of antibodies against *Legionella* SG 1 among healthy blood donors in Latvia (0.2 %), where residing in a multi-apartment building with a centralised hot water supply system was identified as the main environmental risk factor associated with seropositivity, and seroprevalence with regard to *L. pneumophila* SG 2-6 reached 9.5 % among

the inhabitants of multi-apartment buildings in larger cities. The main causative agent of Legionnaires' disease worldwide is *L. pneumophila* SG 1 (ECDC, 2022), and clinical diagnostic tests are therefore targeted to the detection of SG 1. Therefore, it is important to note that the first choice is still urine antigen test, which is specific for infection with SG 1 of *L. pneumophila*, and only 11 % of Legionnaires' disease cases in Europe have been confirmed by methods that require the isolation of bacterial culture (ECDC, 2022), thus it is likely that Legionnaires' disease cases associated with other serogroups are still underreported. Infection may occur by simultaneous contact with several *Legionella* strains that may differ by the elicited immune response and antimicrobial resistance traits, therefore the availability of appropriate diagnostic tests for clinical cases may be of particular importance.

The occurrence of free-living amoebae was considerably higher, with 77.2 % of samples on average containing at least one genus of amoebae. Depending on the sample type, the prevalence of free-living amoebae could reach 95 %. The most often identified were *Acanthamoeba* (54.5 % of all samples) and *Vermamoeba* (28.7 %), followed by *Vahlkampfia*, while more than 20 % of samples contained more than one genus of amoebae, most commonly the combination of *Acanthamoeba* and *Vermamoeba*. Similar results were also reported by other authors who found free-living amoebae in drinking water and environmental samples (Magnet et.al, 2015; Pagnier et al., 2015; Javanmard et al., 2017; Dendana et al., 2018; Üstüntürk-Onan et al., 2018), in biofilms (Declerk et al., 2007; Hsu et al., 2011), industrial waters (Scheikl et al., 2014), and in cooling towers (Scheikl et al., 2016).

The samples obtained during this study always contained *Legionella* together with free-living amoebae and there were no samples containing *Legionella* in the absence of amoebae. The coexistence of *Legionella* with amoebae in water supply systems may indicate greater health risks arising from

the areas of plumbing that are proximal to the points of use, characterised by lower temperatures (Cervero-Arago et al., 2014). In addition to protecting *Legionella* from environmental stress, free-living amoebae may support viable but non-culturable strains (Dietersdorfer et al., 2018) and promote long-term persistence and transmission of *Legionella* (Denoncurt et al., 2018).

Currently there are no Latvian regulations requiring the implementation of risk management plans and regular environmental monitoring regarding *Legionella* spp., and the majority of facility managers are not concerned about *Legionella* risk. Thus, no minimum temperature requirements exist in Latvia for hot water at the point of use. Numerous countries have implemented basic requirements and rules for the prevention of Legionnaires' disease, but the regulations regarding *Legionella* mitigation differ among countries (Kenhove et al., 2019). The majority of guidelines and rules are intended for the control of *Legionella*, while not accounting for the presence of free-living amoebae. The current strategies for *Legionella* control include the identification of *Legionella* spp. via culturing and do not reveal the actual public health risk and burden of *Legionella* infections (Shaheen et al., 2019). The data obtained during this study may help to focus the attention on the common occurrence of *Legionella* and free-living amoebae, their close correlation and persistence, which may be useful for the development of new approaches to water supply safety, taking into account new targets, such as viable but not culturable pathogens and unicellular organisms.

The absence of *Legionella* risk management measures and control procedures may promote further spread of *Legionella* in water supply systems and cause recurring clusters of *Legionella* infections. Furthermore, the frequent occurrence of amoebae that accompany *Legionella* indicates that the traditional monitoring methods may be insufficient for the control of *Legionella*. The development and implementation of additional risk management measures in

order to mitigate the presence of free-living amoebae may substantially enhance the procedures for controlling *Legionella*.

3.3 The genetic diversity of *L. pneumophila*

In this study, full genome sequencing of 137 *L. pneumophila* isolates revealed a great diversity – there were a total of 46 sequence types, of which 14 were endemic to Latvia, as well as ten new, previously unreported sequences were discovered, which have not yet received a sequence type number. Sequence-based typing is still the molecular biology gold standard for assessing *Legionella* spp. strains – both for epidemiological analysis and for the identification of new infection sources, such as air humidifiers, agricultural dust, and damp soil. The broad genetic diversity of *Legionella* spp. has been also described in earlier research, for example, a study from Israel where 78 isolates contained 27 different identified sequence types (Yakunin et al., 2020), a study from Slovenia characterised 88 *L. pneumophila* isolates as belonging to 33 sequence types (Keše et al., 2021), and 141 cases of Legionnaires' disease from Canada were assigned to 57 different sequence types (Levesque et al., 2016). In all of those studies new sequence types were also discovered. The discovery of novel sequence types indicates regionally unique genetic structures of *Legionella* spp. strains, which may substantially differ from previously described clinical or environmental isolates from other countries (Jiang et al., 2021). The recombination and gene transfer between *Legionella* species and strains is the main reason for the great genetic diversity (Herwaldt et al., 2018; Cazalet et al., 2004).

Seven clonal complexes of *L. pneumophila* were identified in this study, which did not carry substantial geographical associations or links to specific serogroups. Only two clonal complexes CC IV and CC VII were formed from sequence types representing one serogroup – SG 1, but CC IV contained only

isolates from the capital city Riga, while CC VII included isolates from the towns of Valmiera, Tukums, and Madona. Similar observations were also made during other studies, where *L. pneumophila* strains obtained from one cooling tower were assigned to separate clonal complexes (Nakanishi et al., 2019; Kozak-Muiznieks et al., 2014).

One of the main advantages of the SBT method is the possibility to compare *L. pneumophila* sequence types to isolates from other parts of the world. The sequence types most frequently encountered in this study were ST-338, ST-366, and ST-1104, where each type was represented by at least 10 *L. pneumophila* strains, and at least 17 other sequence types found in Latvia were also identified by other authors in clinical isolates from sporadic cases, infection clusters, and travel-related cases in various countries of the world (Vekens et al., 2012; Pancer K., 2013; Lévesque et al., 2016; Kozak-Muiznieks et al., 2014, Keše et al., 2021; Sreenath et al., 2020). These precedents give reasons for concern that the *L. pneumophila* strains persisting in the water supply systems of Latvia may create a risk to public health under certain conditions.

A common sequence type found in both clinical and environmental isolates worldwide is ST 1 (Amemura-Maekawa et al., 2010; Tijet et al., 2010; Guo et al., 2015; Mercante et al., 2018), but our study shows a rather different situation in Latvia, because ST 1 was found in only one environmental *L. pneumophila* strain of those 137 for which ST was identified. Other researchers have attributed this to localized expansion of sequence types in the region (Kozak-Muiznieks et al., 2014). For example, the predominant sequence type in several European countries – Belgium (Vekens et al., 2012), United Kingdom (Harrison et al., 2009), France (Ginevra et al., 2008), and the Netherlands (Den Boer et al., 2008) has been ST 47. Furthermore, ST 47 has been found not only in clinical samples and water, but also in soil (Schalk et al., 2014). The sequence type 47 has not been found in Latvia, but data about the

Baltic Sea region are relatively sparse. The nearest study from Poland also did not mention ST 47 (Pancer K., 2013), possibly supporting the interpretation about local genetic variants. A substantial contribution to the studies of *L. pneumophila* genetic diversity and epidemiology could be made by comparing *Legionella* spp. strains isolated from clinical human cases to environmental isolates, yet clinical isolates have not been successfully collected in Latvia, while the standard urine antigen test is suitable only for the diagnosis of Legionnaires' disease caused by *Legionella* SG 1 (Phin et al., 2014). The urine antigen test is also the most frequent choice for the diagnosis of Legionnaires' disease in other countries, and only few sputum or bronchoalveolar lavage samples may be collected for culturing even in the case of infection clusters (Garner et al., 2019; ECDC, 2022). This is one of the reasons why SG 1 is considered to be the main causative agent for Legionnaires' disease (Guyard et al., 2011).

The application of cgMLST genotype visualization in our study showed that the isolates appearing as a single node according to SBT were revealed by cgMLST as different isolates, for example, ST 728, ST 1104, and ST 651, confirming the assumption that the cgMLST typing method providing analysis of 1519 loci ensures a far superior resolution in *L. pneumophila* assay compared to the SBT scheme based only on the analysis of allele profiles from seven loci (Moran-Gilad et al., 2015). Superior resolution of the method may be instrumental for epidemiological investigation, when a link must be established between a clinical case and its possible source (Wüthrich et al., 2019; van Belkum et al., 2007).

A group of scientists in collaboration with the ESGLI team members are currently developing a new typing scheme despite delays due to the COVID-19 pandemic. The application of a cgMLST scheme with approximately 50 genes is anticipated. This will offer the optimal compromise between improvements in

the resolving power and maintaining a good epidemiological match. Simultaneously, NGS methods are being developed, which enable high resolution typing, while omitting the step of obtaining pure bacterial culture, thus providing epidemiological investigation also in cases when isolate cannot be obtained, for instance, in the cases of virulent but non-culturable *Legionella* spp. infections (Domazetovska et al., 2022).

3.4 The virulence potential of *L. pneumophila*

During this study, 420 virulence genes were identified in 137 environmental isolates of *L. pneumophila*, of which 260 genes were found in all sequenced *L. pneumophila* strains. Genes *enhC, htpB, omp28*, and *mip* coding for virulence factors associated with bacterial surface structures were observed in all isolates, indicating that all isolates are capable of adhesion, binding, and entry into a host cell. The largest gene group coding for *T4SS* effectors was sufficiently variable, but we did not find any *L. pneumophila* strain totally lacking this type of virulence genes. The wide range of genes coding for effectors point to the high plasticity of *L. pneumophila* genome and duplication of effectors, which is an important trait of *Legionella* (Best et Abu Kwaik, 2018). Well-established duplication occurred in the case of *SidE* effectors where members of the *SidE* effector family perform similar functions against one and the same host cell target. It is known that *SidE*, *SdeA*, *SdeB*, and *SdeC* catalyse ubiquitination of host cell proteins. Simultaneous blocking of all four of these effectors, but not separately, impaired intracellular growth, which could be restored by inserting just one of them (Ghosh et O`Connor, 2017).

In our study, the *lvh* locus was found in all *L. pneumophila* isolates. The *Icm/dot* genes code for type IVB secretion system and are responsible for intracellular replication, while the *Legionella vir* homologue or *lvh* locus prepares proteins for IVA type secretion system that promotes conjugation and

virulence by interacting with *Dot/Icm* components (de Buck et al., 2007; Kozak et al., 2010). In a study from Greece, the *lvh* locus was found in 93 % of *L. pneumophila* SG 2-14 strains (Katsiaflaka et al., 2016), while in a similar study from Australia the *lvh* family genes were found in 57 % of environmental SG 1 *L. pneumophila* isolates and 78 % of clinical isolates (Huang et al., 2006). The *lvhB2* gene is very important for the infectivity of bacteria following the exposure to low temperature (Ridenour et al., 2003).

A significant difference was found during the initial analysis between the occurrence of *rtxA* positive isolates, compared to other studies. When the genomes were checked against the VFDB database, the *rtxA* gene was absent in all isolates, in direct contradiction to the data from other studies (Sawczyn-Domanska 2021; Sreenath 2020; Zeng et al., 2019), where between 20.69 % and 100 % of *L. pneumophila* isolates were *rtxA* positive. However, all of those studies relied on PCR for detecting the presence of *rtxA* gene. The *rtx1/rtxA-rtx2/rtxA* and *rtx3/rtxA-rtx4/rtxA* primers that are typically used have been developed on the basis of DNA sequence from the *L. pneumophila* strain AA100 (Samrakandi et al., 2002), and they are targeted for only two gene fragments of approximately 540–630 bp length. It is known that *rtxA* itself has modular structure, with highly variable length and sequence similarity between two different *L. pneumophila* strains (D`Auria et al., 2008). Therefore, we proposed that the absence of these two sequences targeted by PCR does not always prove the absence of all possible *rtxA* gene variants.

In order to prove this hypothesis, we simulated PCR analysis *in silico*, using the two aforementioned primer pairs and *L. pneumophila* reference sequences that allowed to characterise the modular structure of *rtxA* (D`Auria et al., 2008). Only the sequence of AA100 strain gave both PCR products *in silico*, confirming the hypothesis. Furthermore, the *rtxA* reference (YP_123037), which was included in the respective VFDB edition, also did not generate any of the

two predicted *in silico* PCR products. Since the *rtxA* sequence from the AA100 strain was the shortest of available references and contained conserved regions located at the start and end of *rtxA*, it was used as a reference for *rtxA* gene screening based on BLAST in *L. pneumophila* genomes. Thus, we can conclude that the method for accurately determining the presence of *rtxA* must be carefully evaluated, taking into account the obvious limitations of PCR methods, as well as alignment-based computational methods and reference databases.

In this study, only one aminoglycoside O-phosphotransferase *aph(9)-la* gene was found in all *L. pneumophila* isolates. This gene is responsible for resistance against spectinomycin, but this result should not be considered as conclusive because spectinomycin is very rarely used for the treatment of Legionnaires' disease (Svetlicic et al., 2023).

The high prevalence, broad genetic diversity, and the wide range of virulence genes observed in all isolates from residential buildings in Latvia mean that all strains persisting in water-supply systems can be considered to be potentially pathogenic. Furthermore, genome rearrangements and gene transfer from various eukaryotic host cells in the environment allow for independent and parallel emergence of new pathogenic strains (David et al., 2016; Gomez-Valero et Buchrieser, 2019).

The evaluation of virulence potential only by the presence of certain genes coding for virulence factors does not provide for clear conclusions because the genome of *Legionella* is very dynamic – it contains a large mobile segment mostly consisting of type IV secretion systems (Gomez-Valero et al., 2014). The tight association of *Legionella* spp. with unicellular organisms and macrophages has resulted in coevolution, and molecular host-pathogen interactions have led to horizontal gene transfer. *Legionella* have acquired eukaryotic-like proteins from all kingdoms of living organisms – plants, animals, fungi, and archaea in an unusually high number and variety (Gomez-Valero and Buchrieser, 2019).

Furthermore, horizontal gene transfer may occur every time when *Legionella* coexist with other strains or species of *Legionella* and other organisms. Although the basic genome is conserved, the exchange of genes coding for virulence has been often observed (de Felipe et al., 2005; Sandeep et al., 2016). This is the major reason for the genetic diversity of *Legionella*, and laboratory tests cannot replicate the same conditions as experienced by bacteria prior to entering alveolar macrophages and, in addition, unpredictable gene transfer might occur during sample processing and concentration steps at the laboratory. For these reasons, different clinical outcomes may be observed even within a single cluster of cases, because the pathogen diversity along with the state of human immune system can determine the clinical outcome (McAdam et al., 2014).

The authors of earlier studies have proposed that only a certain group of environmental *Legionella* spp. isolates can cause disease in humans, and this was also confirmed by comparative genomics research showing that the clinical and environmental isolates of *L. pneumophila* were genetically distinct (Gomez-Valero et al., 2014). In addition, there was much less genetic diversity among clinical isolates, but several studies indicated that the infections in humans cannot be attributed to the propagation of any particularly virulent strains in the environment and the virulence of specific *L. pneumophila* strains is most probably caused by their ability to persist and grow in water supply systems that may serve as sources of infection (Sousa et al., 2018).

The 137 environmental isolates of *L. pneumophila* analysed during our study exhibited a great genetic diversity and substantial presence of key virulence factors, confirming the hypothesis that *L. pneumophila* strains persisting in water supply systems must be viewed as potentially infectious regardless of their serogroup, sequence type, and the set of virulence factors at the time of testing, requiring all preventive and control measures. The infection with *Legionella* occurs by inhalation of water mist contaminated with bacteria,

and the concentration of bacteria may have a key effect on the clinical outcome (Sousa et al., 2018). Therefore, it is clear that the main preventive measure against Legionnaires' disease is the control of *Legionella* spp. proliferation in engineered water systems. The development and implementation of a comprehensive, evidence-based plan for the control of *Legionella* spp. can substantially prevent the risk of infection in humans.

Conclusions

1. The residents of multi-apartment buildings are exposed to a greater risk of *Legionella* infections compared to those living in single-family houses (OR = 2.23; p = 0.011).
2. The highest probability of seropositivity is observed for the residents of buildings receiving centralised hot water supply (OR = 3.16; p = 0.001).
3. The high prevalence of *Legionella* spp. in water supply systems is associated with inadequate hot water temperature (mean value 47.8 ± 0.7 °C) and high occurrence of free-living amoebae (84.2 %).
4. Environmental isolates of *L. pneumophila* show a great diversity of SBT and cgMLST sequence types. There were signs of regional clonal expansion, with a characteristic group of dominant sequence types ST-338, ST-366, and ST-1104 found in our region.
5. The relative frequency of virulence genes indicates that *L. pneumophila* isolates possess high potential for virulence and can be characterised by a widespread presence of virulence factors.

Recommendations

- Taking into account the increased risk of *Legionella* infections for the city residents living in multi-apartment buildings with centralised hot water supply, these buildings should be considered as priority targets for the development and implementation of comprehensive, evidence-based plans for the control of *Legionella* spp.
- Improved testing algorithms for *Legionella* should account for the local abundance of *L. pneumophila* strains and rely on at least two test methods, including also the identification of different SG and genotypes, for example, PCR-based methods and the classic culturing approach.
- The cgMLST method should be introduced in the genotyping of clinical and environmental isolates, providing an accurate and reliable tool for epidemiological investigation.

List of publications on the topic of the Thesis

Publications in international peer-reviewed scientific journals:

1. **Valciņa, O.;** Pūle, D.; Lucenko, I.; Krastiņa, D.; Šteingolde, Ž.; Krūmiņa, A.; Bērziņš, A. *Legionella pneumophila* Seropositivity-Associated Factors in Latvian Blood Donors. *International Journal of Environmental Research and Public Health* **2015,** 13 (1), 58. https://doi.org/10.3390/ijerph13010058.

2. **Valciņa, O.;** Pūle, D.; Mališevs, A.; Trofimova, J.; Makarova, S.; Konvisers, G.; Bērziņš, A.; Krūmiņa, A. Co-Occurrence of Free-Living Amoeba and *Legionella* in Drinking Water Supply Systems. *Medicina* **2019,** 55 (8), 492. https://doi.org/10.3390/medicina55080492.

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2. **Valcina O.,** Pule D., Malisevs A., Trofimova J., Staskevica A., Makarova S., Grantina-Ievina L., Berzins A.; Krumina A. Diversity and factors associated with occurrence of *Legionella pneumophila* in drinking water supply systems. Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge For Use in Practice", 2019, Riga, Latvia

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2. **Valciņa O.,** Krastiņa D., Pūle D., Bērziņš A., Krūmiņa A. Seroprevalence of Legionella in blood donors and occurrence of *Legionella pneumophila* in water distribution systems in Riga. **//** The annual scientific conference of the Riga Stradiņš University, 2015, Riga, Latvia

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Annexes

Decision by the Rīga Stradiņš University ethics committee

Veidlapa Nr. E-9 (2)

RSU ĒTIKAS KOMITEJAS LĒMUMS

Rīga, Dzirciema iela 16, LV-1007
Tel. 67409101

	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Asoc. prof. Olafs Brūvers	Dr.theo.	teologs
2.	Profesore Vija Sīle	Dr.phil.	filozofs
3.	Docente Santa Purviņa	Dr.med.	farmakologs
4.	Asoc. prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5.	Profesore Regīna Kleina	Dr.med.	patalogs
6.	Asoc. prof. Guntars Pupelis	Dr.med.	ķirurgs
7.	Asoc. prof. Viesturs Liguts	Dr.med.	toksikologs

Pieteikuma iesniedzējs: **Olga Valciņa, doktorante**
Medicīnas fakultāte

Pētījuma nosaukums: „Legionella pneumophilia molekulārā epidemioloģija"

Iesniegšanas datums: 30.10.2013.

Pētījuma protokols: Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot ar dalībniekiem (bez kāda apdraudējuma veselībai) klīnisko paraugu ņemšanu un laboratoriskās analīzes, kā arī vides paraugu analīzi, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

Izskaidrošanas formulārs: ir

Piekrišana piedalīties pētījumā: ir

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., asoc. prof.

Paraksts

Ētikas komitejas sēdes datums: 31.10.2013.

Study participant survey form

Anketas numurs: _____

Datums:_____ Intervētāja kods_____

„*Legionella pneumophila* molekulārā epidemioloģija"

Asins donoru seroprevalences pētījuma dalībnieka anketa

Labdien!

Esam Jums pateicīgi, ka piekritāt piedalīties pētījuma „*Legionella pneumophila* molekulārā epidemioloģija", kurā tiks iegūti dati par antivielu pret Legionella pneumophila prevalenci Latvijas asins donoru vidū. Pētījuma sadaļas mērķis ir novērtēt seropozitivitātes asociētos faktorus, t.sk. tiks ņemti ūdens paraugi dzīvojamās ēkās, kuros tiks noteikta Legionellu klātbūtne un skaits.

Paldies, ka veltīsiet tam laiku – Jūsu sniegtā informācija būs ļoti noderīga Legionella spp. ierobežošanas stratēģijas izstrādei. Jūsu atbildes un analīžu rezultāti tiks izmantoti pētījumā tikai apkopotā veidā.

1. Dzimums

☐ vīrietis ☐ sieviete

2. Vecums, (pilni gadi)_____

3. Vai Jūs dzīvojiet privātmājā: ☐jā ☐nē

4. Dzīvesvieta

Apdzīvota vieta, pilsēta, ciems, novads _____

Iela _____

Ja dzīvojiet daudzdzīvokļu ēkā, lūdzu, norādiet ēkas numuru _____

Kurā gadā celta ēka_____

Vai bijusi ūdensvada renovācija ☐jā ☐nē

Pasta indekss_____

5. Karstā ūdens apgādes veids Jūsu mājās:

- ☐ Centralizētā apgāde
- ☐ Elektriskais boileris
- ☐ Gāzes caurteces sildītājs

Cits: _____

6. Vai Jums ikdienā ir regulāra saskarsme ar ūdens aerosoliem (darbs ar izsmidzināšanas sistēmām, rūpnieciskām un medicīnas iekārtām, strūklakām, burbuļvannām u.tml.)

☐jā ☐nē

Ja atbilde bija jā, lūdzu, norādiet, vai izmantojiet aizsargmasku:

☐jā ☐nē

7. Vai Jūs smēķējat? ☐jā ☐nē

Ja jā, tad norādiet vidējo cigarešu skaitu dienā_____

8. Vai esiet slimojis (slimojusi) ar Leģionāru slimību?

☐jā ☐nē

9. Vai pēdējā gada laikā Jūs esiet slimojis (slimojusi) ar:

Pneimoniju (plaušu karsoni) ☐jā ☐nē

Bronhītu ☐jā ☐nē

Gripai līdzīgu saslimšanu vai citām augšējo elpceļu slimībām ☐jā ☐nē

10. Vai pēdējā gada laikā Jūs esiet bijis dušā ārpus mājām ☐nē

☐jā viesnīcā ☐jā ciemos ☐jā darbā ☐jā slimnīcā

Paldies par Jūsu veltīto laiku! Mēs to ļoti novērtējam!