Prevalence and diversity of *Legionella* colonization in built aquatic environments

PhD thesis

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Introduction

In the past decades, the dynamics of water related diseases has changed considerably in the developed countries. While classic waterborne outbreaks have practically disappeared as a consequence of wastewater treatment and improved hygiene conditions, new pathogens emerged which can resist the current water treatment practices. The faecal indicators used for water quality assessment do not predict the presence of these pathogens, thus in the absence of targeted analysis the risk is unobserved until the emergence of infections.

Legionellae are the etiological agents of Legionnaires' disease, an often severe pneumonia. They are present in most natural water environments. However, they predominantly pose a risk of infection in built water systems where conditions are favourable for their proliferation.

The inhalation of aerosol generated from *Legionella* contaminated water is the primary mean of transmission. There is thus a risk of infection everywhere where water droplets of 1-3 µm diameter are generated. An increasing number of studies have shown that the role of domestic hot water systems and bathing pools, as potential sources of infection, is underestimated.

The number of diagnosed legionellosis cases is relatively low. In Europe, on average 12 cases/1 million inhabitants are reported yearly, in Hungary, only half of this number. However, this is most likely only a fraction of the actual cases. In the developed countries, pneumonia is the 10th leading cause of death, and the first between the infectious diseases. In Hungary, the estimated number of pneumonia incidents is 2000 per 1 million inhabitants, and in the most cases the causative agent remains unidentified. Among the hospital-acquired infections, pneumonia poses also the highest risk.

In general, countries only recognize the relevance of the problem after the first major outbreak, and develop the regulation for environmental risk assessment and limit values as a consequence. International guidelines usually define 1000 colony forming units (CFU)/L for domestic water systems and 100 CFU/L for bathing pools as limit value of public health concern.

In Hungary, until recently there was no regulation for environmental *Legionella* monitoring (neither limit value, nor testing obligation). In absence of any legal background, the operator were not required to carry out *Legionella* testing, and thus they generally did not have any information about the *Legionella* colonization of the operated facilities and the influencing factors. Without appropriate knowledge the operators did not practice proper water safety practices. For the same

reason the available previous data on *Legionella* prevalence in domestic water systems was very limited. The current study is the first comprehensive work on this subject.

Legionella prevalence can be tracked to technical rather than hygienic problems. The proper design and operation of domestic water systems and pools are essential to prevent or reduce the *Legionella* colonization and to minimize the risk of infection. The good operating practices specific for the Hungarian water systems can only be determined, if we know the effect of various parameters on *Legionella* colonization.

Aims

The aim of the present study was to assess the environmental prevalence of *Legionella* and the associated public health risk in Hungary, to analyse the means of risk management, and subsequently support the development of a legal regulation. The objectives of the work were the following:

- To sample cold and hot water systems of various buildings and pools, realizing the greatest possible diversity in the selection of the buildings sampled, both geographically and regarding building type.
- To investigate the *Legionella* prevalence in the cold and hot water systems various buildings'.
- To characterize the *Legionella* strains isolated from cold and hot water systems and pools.
- To establish methods for the identification and rapid and efficient separation *Legionella* isolates and to test the selected methods for epidemiological purposes.
- To investigate the relationship between those parameters (including building characteristics and basic water quality data), which are known or easily available to building operator, and the *Legionella* count of the water samples with statistical methods. Those parameters are: source water, water temperature, technical parameters, biotic parameters (heterotrophic plate count and *Pseudomonas aeruginosa* count) and building type.
- To carry out risk assessment of settings previously associated with Legionnaires' disease cases.
- To control the effectiveness of interventions carried out for the risk mitigation.
- To compile a risk assessment questionnaire for hot water systems and pools based on the experiences of the site visits and the risk assessments carried out previously.

Materials and methods

In total, 1809 hot water and 449 cold water samples were taken from the hot water storage tanks, showerheads and tap faucets of different type of buildings (168), such a health care facilities (22), accommodation sites (21), educational institutions (26), office buildings (10), industrial buildings (35) and private residences with central (26) and individual hot water supply (26) between January 2006 and June 2013. Further 146 pool water samples were taken from 29 different facilities. Most of the pools operated in hotels (13) and in private residences (6).

Water samples were collected according to the standard MSZ EN ISO 19458:2007; cold water samples were taken after 2 minutes, hot water samples after 1 minute flushing. Water samples were collected under normal operating conditions (i.e. during working days in office buildings). The sampling scheme within the buildings was designed to represent the entire domestic water system. The pool water samples were taken from those part of the pool where the contamination was expected the highest and where the where the concentration of the disinfectant residual was constant.

The water samples were processed, incubated and the results evaluated according to the standard MSZ EN ISO 11731-2:2008, briefly as follows: 10 or 100 mL aliquot was vacuum filtered and washed with acid buffer. *Legionella* sp. was cultured on GVPC at 36 ± 1 °C for 10 days. Results were read under a dissecting microscope. Presumptive *Legionella* colonies (at least 3 colonies per plate) were subcultured on BCYE with and without cysteine to test for cysteine auxotrophy. Presumptive *Legionella* sp. strains were identified by seroagglutination. The test allows for the differentiation of *Legionella pneumophila* serogroup 1 and 2-14 and the detection of seven species of non-*pneumophila* legionellae. *Legionella* genus-specific PCR (from cell suspension) was used to confirm the genus level identification. Preliminary clustering was carried out with REP-PCR and/or with MALDI-TOF mass spectrometry. Group representative strains were typed to serotype or MAb-type level using other antigen based typing methods of higher resolution than latex agglutination. *P. aeruginosa* and heterotrophic plate count were determined according to the related standards (MSZ EN ISO 16266:2008 and MSZ EN ISO 6222:2000).

The most important technical and other risk factors were recorded using a risk assessment questionnaire. Those well-characterized water distribution systems were included in the analysis, where the samples represented the water quality of the whole system. Chemical characteristics of the supplied water were derived from the National Drinking Water Database.

Statistical analysis was performed using SPSS. Mann Whitney test (for 2 groups) and Kruskal Wallis test (for 3 or more groups) were performed to compare the mean values of the measured variables in connection with *Legionella* spp. Colonization. Chi square and Fisher exact test were used for the comparison the *Legionella* diversity in the different groups. The following variables were taken into consideration: drinking water source, age, complexity and size of the building, age of the premise plumbing system, hot water temperature, temperature decrease within the hot water network, production and storage of hot water including individual or central production, type of primer heat, the connection and position of storage tanks, volume of stored water, presence of circulation, distance from the heat exchanger, indicator organisms (heterotrophic plate count and *P. aeruginosa* count).

To identify the independent risk factors that determine the *Legionella* contamination in hot water systems using centrally produced hot water, the variables that were significant in the non-parametric tests (e.g. water temperature) were analysed further to assess the strength of their association with *Legionella* positivity. The variables were dichotomized (e.g. water temperature below/above 55 ^{o}C) and tested by univariate logistic regression, and the significant variables from these tests were included in a multivariate analysis. Correlation of *Legionella* counts and chemical parameters was assessed using regression analysis for the determination of significance levels.

Results

1. The present study is the first survey in Central-Europe on the *Legionella* prevalence in domestic water systems. Studies of similar size are rare in the international literature. Unlike most similar studies, analysis was not limited to one building type, but all facilities were sampled where there is a risk of *Legionella* exposure from the domestic water system. Thus schools, office buildings and industrial facilities were also included in the study; previous systematic prevalence study for these facilities is not available in the scientific literature. The survey – unlike most studies – covered both hot and cold water systems.
2. The extensive colonization of domestic cold and hot water systems in health care facilities, accommodation sites and residential building was confirmed; its extent was shown to be similar or higher in Hungary than in the previous international observations. The rate of *Legionella* prevalence was highest in the hot water system of health care facilities (91.7 %), and lowest in the residential buildings with individual hot water production (7.4 %). *Legionella*

was detected in 71.5 % of the accommodation sites, and 71.6 % of the residential buildings using central hot water production.

3. Schools, industry facilities, and office buildings are similar to other, better recognised risk environments (such as hospitals and hotels) in this regard, and the importance of risk management in these setting should not be overlooked (rate of colonization respectively 53.8 %, 77.1 % and 60.0 %).
4. It was confirmed that the colonization rate of buildings with centrally produced hot water was in all building types high (rate of positive samples was 49.2 %), which indicates that the risk of infection is primarily determined by the likelihood and type of exposure and the susceptibility of the people.
5. Our results show that in most hospitals immediate intervention is needed due the high rate of the colonization and the increased sensitivity of the patients.
6. Individually produced hot water was found to be lower risk than centrally produced hot water (rate of positive samples 16.3 %), though in some individual hot water storage systems extreme *Legionella* counts were observed ($3.2.10^3$ – $1.5.10^6$ CFU/L). Thus in contrast to the international guidance it was assumed that individual hot water systems using hot water storage may also pose a health risk and risk assessment should be extended to these systems.
7. The first complex methodology was introduced in Hungary for the detection, identification and typing of *Legionella.* The methodology is suitable for rapid, cost- and resource-efficient environmental investigation of outbreaks, clustering of the isolates, identification of the strain responsible of the infections and comparison of the environmental and clinical isolates (where available). In case of the environmental *Legionella* isolates, *Legionella* genus specific PCR was used for genus level and antigen based identification of *L. pneumophila* species (Oxoid latex agglutination). The latter method was performed for each isolates. In case of epidemiological investigations, often 100 or in same cases several hundred *Legionella* strains are isolated, thus the preliminary clustering is indispensable. REP-PCR and/or MALDI-TOF mass spectrometry was used for generating clusters, and group representatives were typed further by antigen based methods (microagglutionation and/or MAb typing) which are more sensitive, but also more cost- and resource intensive than latex agglutination. The results were used for selecting the strains for comparison with clinical isolates (using PFGE or MLST methods).
8. The results have confirmed the dominance of *L. pneumophila* 2-14 in the hot water systems of Hungary. Most water systems are colonized by more than one *Legionella* strains. The most

prevalent serotypes were *L. pneumophila* 6, 10, 3, and *L. pneumophila* 1, especially the MAb 3/1 negative „Bellingham" and „OLDA", and the MAb 3/1 positive „Philadelphia".

9. *L. pneumophila* 1 serotype was isolated in higher ratio from health care facility water systems than in other building types (the rate of *L. pneumophila* 1 among the positive samples was 32.0 % in total, while 48.1 % in the hospital samples). Comparative analysis revealed that this was especially true for hospitals reporting legionellosis incidents. Thus the presence of *L. pneumophila* 1 is a confirmed risk factor.
10. Spa pools were shown – in a non-representative study – to represent the highest risk setting among pools, not only because of the increased exposition, but also due to their higher colonization rates (33.8 % positive compared to 7.5 % of other pools). Negative pool water result was demonstrated to be an insufficient indicator of the colonization of the system, thus it was proposed to also analyse the filtered pool water regularly.
11. The effect of various parameters on *Legionella* counts in hot water systems was analysed on a sample size meeting the largest previous international studies.
12. The major determinant of *Legionella* colonization in central hot water systems was water temperature. Water temperature above 55 °C was confirmed to be the most protective, however, over 50 °C the risk is greatly reduced. The odds ratio of *Legionella* positivity in samples below 55 °C is over six (OR=6.4); if the confounding factors are removed in multivariate analysis, it is 13.3.
13. This was the first extensive *Legionella* prevalence study where four different potable water sources were compared to analyse the correlation of the water source and the *Legionella* counts in the hot water samples. Bank filtration and carstic water were not assessed previously. Groundwater derived hot water was demonstrated to be lower risk than more vulnerable sources (OR: 3.6). This result is more likely to correspond to the microbiology than the chemistry of the source water, as the effect of the latter was shown to be minimal. Our hypothesis is that the legionellae present in the raw water survive water treatment in very low numbers and proliferate under favourable conditions. The difference was independent of water treatment and disinfection.
14. The correlation of size of the hot water system and *Legionella* colonization was assessed for the first time. The result was significant even in multivariate analysis. The probability of *Legionella* positive samples is twice as high if the hot water is produced outside of the building (OR: 2.0).

15. Risk assessment was performed in the buildings and pools associated with legionellosis incidents, using the study results and supporting the public health authorities. In four cases, the relationship of the water system (hot water or pool) derived and the clinical *L. pneumophila* 1 strains was confirmed.
16. The findings of the current study supported the development of the regulation on the "Hygiene requirements of matrices and facilities presenting *Legionella* infectious risk" and the corresponding guidance document. The guidance document provides risk management suggestions for the affected facilities. In those facilities, where long-term monitoring was possible, risk management interventions were usually successful; *Legionella* counts were reduced in all investigated buildings.

Conclusions

It is little known in Hungary – even among technical and public health professionals – that legionellae are widely prevalent in built water environments and legionnaires' disease is regarded an "air-conditioning disease". International experience shows that Legionnaires' disease only gains public awareness after major outbreaks. There was no such outbreak in Hungary, most of the legionellosis cases are not publicly known. The number of reported cases does not represent the scope and the problem, and it is most likely only a fraction of the actual cases. The etiological agent of most community acquired or health care associated pneumonia cases is not identified, thus the ratio of *Legionella* infections cannot be estimated. The correct assessment and management – sometimes even in the presence of infections – is often hindered by economic considerations, indifference or ignorance.

The actual situation is difficult to reveal, since a case illness or an outbreak associated with the facility – especially the highly affected hospitals and hotels – is a strong negative message that the operators wish to avoid. Recently, it was in the headlines that *Legionella* was detected in a university hospital. The incident generated wide interest, although according to our investigation, it is less likely that a hospital in Hungary is negative for *Legionella.* While the presence of *Legionella* in water systems may remain unknown until the emergence of infections, risk management interventions are often very visible: the case mentioned above was revealed after first the use of the hospital's whole water system, then the hot water system was restricted until the other interventions were carried out. The above facts underline the importance of the current study in assessing the actual prevalence.

The risk of *Legionella* proliferation is mainly dependent on engineering parameter, as our study has also shown. Risk management therefore starts in the design phase. Since often the engineers are unaware of the risk, hospitals are still being built with 43 °C hot water circulating in the entire system. The best practice of operation can be exactly defined, as it is detailed in the current study.

Based on the experience from the investigation of water systems, a risk assessment questionnaire was developed. The questionnaire covers all matrices where legionellae can proliferate and aerosol may be generated thus creating a risk of infection. The questionnaire assists both the operators and the public health authorities in assessing the operation practices and subsequently the *Legionella* risk. Though water analysis for *Legionella* gives more direct information on the rate of colonization, it cannot be the sole tool of *Legionella* risk assessment.

In the absence of legal regulation, adequate standards or best practice guidance even the interpretation of water analysis result was difficult for the operators, and until recently there was no guidance available on risk management options. It was the responsibility of public health and work safety professionals to support the operators in risk management, and the first step was the development of the national regulation supported by best practice guidance. On of the major achievement of the current work is that it was partially the basis of the Decree of the Minister of Human Resources published in November 2015, on the public health requirements of matrices and facilities posing a risk of *Legionella* infections. The aim of the regulation is to prevent extensive outbreaks (up to several hundred cases) like the recent one in Spain. Efficient implementation of the developed regulation and sufficient information to all stakeholders it is possible the prevention of such outbreaks.

Publications

Publications in connection with the thesis

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