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Persistent colonization of 2 hospital water supplies by *L. pneumophila* strains through 7 years – Sequence-based typing and serotyping as useful tools for complex risk analysis

Katarzyna Pancer[1], Renata Matuszewska[1], Marta Bartosik[1], Krzysztof Kacperski[2], Bożena Krogulska[1]

[1] *National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland*
[2] *Center of Oncology – Institute, Warsaw, Poland*

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Abstract

Contamination with *Legionella* spp. of hot water system (HWS) in hospitals is a considerable problem and elimination of bacteria poses difficulties. Obligatory control of *Legionella* spp. in hospital HWS was implemented in Poland in 2008y. After that, *Legionella* spp. has been isolated repeatedly from HWS of the majority of hospitals. The aim of our study was to confirm the permanent colonization with Legionella spp. of 2 hospital HWSs based on the antigenic (serogroup/subgroups) and genetic properties (SBT, *rtx*A) of *L.pneumophila* strains isolated in 2004–2011. The dynamic of *L.pneumophila* population was also examined due to methods of disinfections applied during 7 years. Totally, 134 environmental samples were collected from two hospitals in 2004–2011 (118 from HWSs). During the study disinfection by chlorine dioxide was implemented in both hospitals, while thermal shock was added in the hospital A. Isolated *L.pneumophila* were serogrouped (105 strains) using Dresden MAb Panel, genotyped by sequence based typing (53) and by harboring of *rtx*A gene (58 isolates). *Legionella* spp. were still presented in both systems after 7 years. Exactly the same strains (ST1, ST87, ST114, ST992) were found in the hospital B. While changes of *L.pneumophila* population were observed in the hospital A: strains still occurred after 7 years (ST835 Sg6, ST114 Sg6); modified antigenic properties (ST835 – Sg12 vs. Sg6); eliminated or maybe not detected (ST81, ST838, ST959). Moreover, the majority of examined strains ST1 (Sg1, OLDA) harboured *rtx*A gene (hospital B). Our results and data in the EWGLI SBT base indicated higher risk of *Legionella* infection in the hospital B than A – because of heavy colonization with *L.pneumophila* ST1. The risk assessment of *Legionella* infection based only on technical parameters, extent of colonization/contamination level may be not completed. It should be supplemented with the additional examination: serotyping, genotyping and virulence testing of isolated strains.

Key words

Legionella, persistent colonization, risk of nosocomial legionellosis, serogrouping

INTRODUCTION

Bacteria *Legionella* are ubiquitous in domestic hot and cold water systems of the healthcare facility. The *Legionella* species can be present in tap waters, hot water tanks, shower heads, air conditioning systems or humidifiers within/outside the building. Several reports have shown an association between the presence of legionellae in water from nebulizers, other medical respiratory equipment or for hydrotherapy and the occurrence of *Legionella* infection [1, 2, 3, 4, 5, 6, 7, 8, 9]. Temperature of water, especially between 25–45°C, can support the growth of these organisms. In addition, the presence of nutrient sources, biofilm or slime layers containing other bacteria, protozoa and algae, stagnation and dead-legs favour the occurrence of *Legionella* spp. [2, 7]. Such conditions create a risk of human infection with these bacteria, especially for hospitalized and/or immunocompromised patients, but also disease among the medical staff cannot be ruled out. If the water plumbing system maintenance is not appropriate, the risk of *Legionella* colonization increases [3, 10].

Clinical manifestations of legionellosis, disease caused by *Legionella* spp., varied from influenza-like, self-limited infection (like Pontiac fever) by severe pneumonia (*Legionella* pneumonia, Legionnaire's disease, LD) to multiple organ failure. The most common forms are influenza-like infection (~90% of all *Legionella* infections) and pneumonia (3–8%) [11]. In 1987, the European Working Group for Legionella Infections (EWGLI) was founded for the control and prevention of Legionnaire's diseases in Europe, especially cases associated with traveling. Non-pneumonia forms of legionellosis are not reported in the majority of the EWGLI countries, however in Poland all cases due to *Legionella* spp. have been obligatory reported since 2002. According to the EWGLI data (ELDSNet – since 2010y.) 80–90% of reported cases of *Legionella* pneumonia (Legionnaire's disease, LD) were caused by bacteria *L. pneumophila*, mostly by *L.pneumophila* serogroup 1 (50–70% of all reported

Address for correspondence: Katarzyna Pancer National Institute of Public Health – National Institute of Hygiene, Warsaw, Chocimska 24, 00-791 Warsaw, Poland
e-mail: kpancer@pzh.gov.pl

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LD cases). The other species belonging to *Legionellaceae* (*L. micdadei, L. longbeache, L. dumofii, L. bozemanii* and others) are associated with 5–20% of all reported cases of *Legionella* pneumonia [1, 6, 7, 12]. The *Legionella* infection is not spread between humans, but the mode of transmission is aerosol of contaminated water [1, 6]. This is a reason why the environmental conditions play a crucial role in the reduction of risk of *Legionella* infection, especially when immunocompromised persons are exposure. It was estimated that 0.1% – 5% of exposed immunocompetent persons and 0.4% – 14% of immunocompromised patients develop pneumonia due to *Legionella* spp. The mortality among patients with Legionnaire's disease varied, depending on the category of infection: travel associated (3% – 5%), community acquired pneumonia (5–15%) and hospital acquired pneumonia (up to 50%). The highest risk of *Legionella* infection was observed among patients hospitalized on the Intensive care unit, after organ transplantation, treated with steroids, chemotherapy as well as patients of naturopathy centres. In Europe and the U.S. over 20% of reported cases of nosocomial legionellosis were diagnosed in patients hospitalized in intensive care units [1, 4, 10, 13].

The control of *Legionella* spp. in water systems is prescribed in Ordinance of the Minister of Health of March 29th, 2007 in Poland [14]. Implementation of these regulation requires testing *Legionella* spp. in hot water samples collected from hospital and other health facilities objects. It was found that in many hospitals *Legionella* spp. has been isolated many time from water samples in each of the following tests.

OBJECTIVE

The aim of our study was to show presence of permanent colonization with *Legionella pneumophila* strains of water distribution systems in two hospitals (or eventually denial of this thesis). The study was based on the results of analysis of occurrence *Legionella* spp. in water samples collected during 7 years, and the antigenic and genetic properties of isolated strains. The dynamic of occurrence of particular strains of *L. pneumophila* was also examined due to methods of disinfections applied during 7 years (2004–2011).

MATERIAL AND METHODS

Hospitals. The study was undertaken in 2 large hospitals (over a 500-bed). Hospital A is a multi-storey building built in the 70's, in which two water supply zones in use are distinguished: the first zone from the basement to the 4th floor, with an average water consumption 9,5–12 m^3 per day, the second zone from the 5^{th} floor to 9^{th}, with an average water consumption of 7.4 m^3 per day. The following work presents results connected with the first zone of water supply. Hospital B is located in two buildings: one that was built in the 30's of the twentieth century and second – new modern building. All research were connected with water supply from the old part of hospital buildings.

Water distribution systems. Domestic installations of both hospitals, are made of different kinds of materials such as steel and plastics. Hot water is stored in tanks, made of corrosion-resistant steel. In not appropriate design and extensive construction of hospital's distribution water systems enhance the microbiological risk. Moreover lack of water temperature control increases the risk of *Legionella* spp. growth. Furthermore, a large amount of sludge, scale and corrosion occurs in the water distribution system. The source of the water is underground water and city water – pipe network.

Environmental testing for legionellae. A total of 134 environmental samples were collected from water supplies of the two hospitals between 2004–2011. The hot water samples (90) and cold water samples (16) were taken from the hospital A, and the hot water samples (28) were taken from the hospital B. Water samples (1 L) were collected in a sterile plastic bottle containing 1mL of 0.1N sodium thiosulfate to neutralize chlorine disinfectant.

Isolation and identification of *Legionella* spp. from water samples was according PN-ISO 11731: 1998 and PN-ISO 11731–2:2008 method [15]. The 1L sample was filter concentrated by pouring the sample into a sterile 47 mm ester cellulose. Additionally, sample were acid treated in equal volume of HCl-KCl acid buffer (pH 2.2) for 5 min. After concentration of a water sample, filters were inoculated on a plate BCYE agar with GVPC selective supplement (Oxoid) and incubated at 36+/-2 °C for 10 days. The plates were examined after 72 h to 96 h (4 to 7 d) incubation. Suspected colonies were aseptically picked and streaked onto BCYE agar plate without L-cysteine [BCYE(–)]. Colonies that can grow on BCYE agar, but not BCYE(–) agar, were considered presumptive *Legionella* species. *L. pneumophila* isolates were first serogrouped with a Latex agglutination test commercial kit (Oxoid) according to the manufacturer's instructions.

Characteristics of isolated *L. pneumophila* strains. Isolated and determined as *L. pneumophila* strains were stored in -70 °C. For the antigenic examinations strains were randomly selected (5–10 strains per water sample). For the genetic examinations strains were selected according to their antigenic properties and the participation of particular serological type in the water sample/water distribution system.

Serotyping: More detailed serotyping examinations were done using The Dresden MAb Panel [16] kindly provided by Dr Jurgen Helbig from the Institute of Medical Microbiology and Hygiene, Medical Faculty of the Technical University Dresden, Germany. The Dresden Panel is an EIA test, in which all serogroups of the *L. pneumophila* strains and subgroups of *L. pneumophila* Sg 1 strains are identified by 26 monoclonal antibodies (MAb). Finally, 105 strains of *L.pneumophila* were serotyped.

Sequence based-typing (SBT): For the purpose of genotyping strains of *L. pneumophila* were selected. Briefly: the number of *L. pneumophila* strains selected for genotyping belonging to particular serogroups and subgroups was based on the proportion of serogroups/subgroups of all tested isolates from this specific water distribution system during that water samples collection. This rule was used in both tested hot water distribution system (HWS). In total, 53 strains of *L.pneumophila* were examined: 30 isolated from HWS of hospital A and 23 isolated from HWS of hospital B.

DNA extraction, PCR amplification. The strains stored at -70 °C were inoculated on BCYEα plates and incubated at 36+/-2 °C for 2 days. Genomic DNA was extracted from selected strains using the QIAamp DNA Blood mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's guidelines. The PCR was performed on C1000 TM Thermal Cycler (BioRad, Polska Sp.z o.o.) using GoTaq Flexi DNA polymerase (Promega, Madison, USA).

SBT. Genotyping was performed according to the seven-gene protocol from the EWGLI SBT scheme http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php previously described [17, 18]. For each isolate, the profile of seven alleles at each of the loci was defined in the following order: *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*. ST was represented by a number.

Presence of locus *rtxA* by PCR. For determination of gene *rtxA* in *L. pneumophila* strains primers previously described were used [19]. The PCR was performed on C1000 TM Thermal Cycler (BioRad, Polska Sp.z o.o.) using GoTaq Flexi DNA polymerase (Promega, Madison, USA). Finally, 58 strains of *L. pneumophila* were tested for *rtxA* gene presence.

RESULTS

The study shown the persistent colonization with bacteria *Legionella* in hot water systems of the both hospitals, and in the cold water system of the hospital A also (Fig. 1). The examinations of water samples collected from the hospital A, showed the presence of *Legionella* spp. in 76.7% of the hot water samples and 75% of cold water samples. A high number of bacteria 1.7×10^2 cfu/100 ml and 1.0×10^3 cfu/100ml has been observed in two cold water samples, whereas in the remaining cold water samples *Legionella* spp. number ranged from 1 cfu/100ml to 77 cfu/100ml. In the all hot water samples collected from the hospital B, *Legionella* spp. has been found. The cold waters samples were not collected.

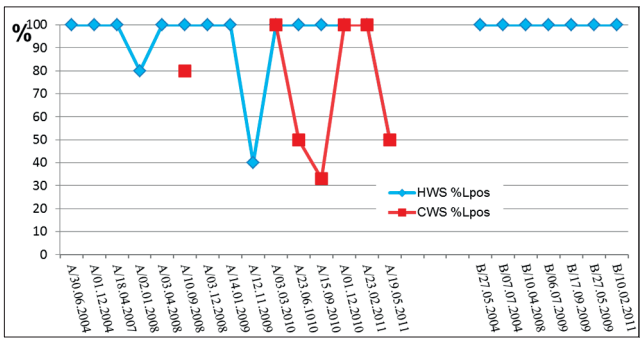

Figure 1. Percentage of Legionella-positive water samples (%Lpos) in cold (CWS) and hot water systems (HWS) in two hospitals (hospital A and B) – by the date of water samples collection

The main problem was to maintain the appropriate temperature of the hot and cold water in the water systems of the both hospitals. The hot water temperature fluctuations was exemplified by the hospital A (Fig. 2), where the measured temperature ranged from 26 °C to 65 °C. The temperature of cold water (hospital A) ranged from 14 °C to 31 °C (Tab. 1).

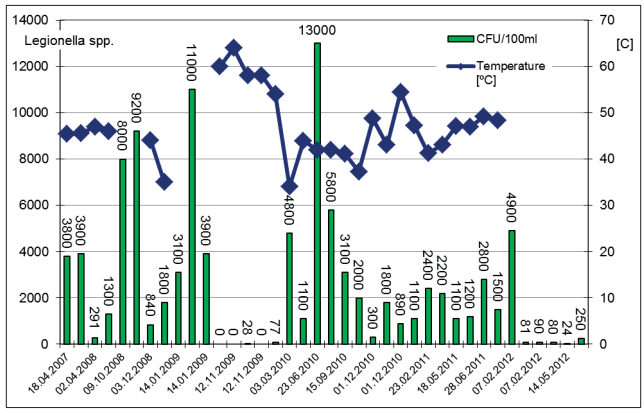

Figure 2. Number of *Legionella* spp. in hot water samples taken from the hospital A in the years 2004-2011, and temperature of hot water samples.

Table 1. The results of *Legionella* spp. tests of cold water samples taken in the hospital A.

Data test	Number of tested samples	Number of *Legionella*-positive samples	Number *Legionella* sp. [cfu/100 ml]	Serogroup	Temperature of water [°C]
10.09.08	5	4	$12–1.0 \times 10^3$	*L. spp.*	14–25
The constantly chlorine dioxide disinfection – started November 2010					
01.12.10	1	1	4	2–14	21
03.03.10	2	2	2–45	2–14	22–27
23.06.10	2	1	69	2–14	25–28
15.09.10	3	2	1–7	2–14	28–29
23.02.11	1	1	1.7×10^2	2–14	29
19.05.11	2	1	9	*L. spp.*	30–31

The cleaning and disinfection procedures were implemented in the hospital A. The thermal disinfection (heating water to a temperature of 70–80°C) was applied first. It was performed first time in the November 2008, next time – twice in 2010 (Fig. 3 – arrows indicate the time of the thermal disinfection). Control tests were performed approximately up to 1 and 2 weeks after thermal disinfection, and the obtained results showed persistent occurrence of *Legionella* spp. in water (Fig. 3). Lack of effects of thermal disinfection repeatedly carried out in November 2010, caused that the continuous running water disinfection with chlorine dioxide

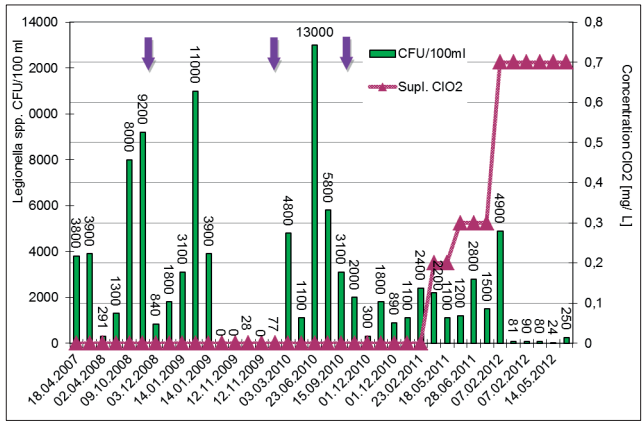

Figure 3. Determined number of *Legionella* spp. (cfu/100 ml) and implemented methods of disinfection: thermal (arrow) and chemical (by concentration of ClO_2) in HWS of the hospital A.

system was implemented. Studies performed periodically to six months after installing the generator ClO_2 showed that in this hospital still occurred *Legionella* spp. in both: the hot water samples – above 10^3 cfu/100 ml, and in the cold water samples – to 9 cfu/100 ml (Fig. 3). Originally, the chlorine dioxide concentration was within the range of 0.1 – 0.3 mg/l, but in the absence of a strong reduction of the number of *Legionella* spp. in water samples, the concentration of chlorine dioxide was increased to the maximum limit (0.7 mg/l) in the January 2012. The subsequent studies identified the presence of *Legionella* spp. in water samples but number of these bacteria was less than 100 cfu/100ml

Implementation of the constantly chlorine dioxide disinfection system has significantly reduced the level of cold water contamination with *Legionella* spp., but it was not successful to eliminate of *Legionella* spp. from the system.

Among the *Legionella* – positive water samples taken from the water supply of the hospital A nearly 94.2% of the samples exceeded the permitted number of bacteria (100 cfu/100 ml), as defined in the Ordinance of the Minister of Health on the quality of water intended for human consumption from March 29, 2007 [14]. The number of *Legionella* spp. exceeds the value of 10^3 cfu/100 ml in the majority of these samples (68.1%), and ranged from 1.1×10^3 cfu/100ml to 7.5×10^3 cfu/100ml. It indicated a high level of hot water contamination in the hospital A. Medium level of contamination was observed in 20.3% of water samples in which the determined number of *Legionella* spp. ranged from 1.7×10^2 cfu/100ml to 9.3×10^2cfu/100ml. A very high contamination of water was recorded only in 4 samples (5.8%), number of bacteria exceeded 10^4 cfu/100ml.

In the hospital B, the presence of *L. pneumophila* was detected in all samples taken from the hot water supply. Bacteria *Legionella* spp. were detected in a high number: from 1.2×10^2cfu/100 ml to 1.9×10^4cfu/100 ml. The water pollution of all tested samples was observed and contamination assessment of system was estimated respectively as low, medium and high/very high (Tab. 2). Relatively low temperature of hot water was noted, from 29°C to 48°C, similarly as in the hospital A, in only a single case it was 55°C (Tab. 2).

Legionella spp. strains isolated from HWS or CWS of the hospital A were identified as *Legionella pneumophila* Sgs 2–14. The most dangerous epidemic *L. pneumophila* Sg 1 was not detected in the *Legionella* – positive water samples. In the hospital B strains of *L. pneumophila* Sg 1 were found in 2004 and 2011y.

Results of phenotyping and genotyping of *L. pneumophila* strains isolated from HWS of 2 hospitals. Totally, 105 strains of *L. pneumophila* were serotyped and subgrouped with monoclonal antibodies: 61 were isolated from the hot water distribution system in the hospital A and 44 – in the hospital B. Bacteria *L. pneumophila* were isolated from 19 water samples collected in years: 2004, 2007, 2010 and 2011 in the hospital A; while strains of *L. pneumophila* were isolated from 13 water samples collected in years 2004 and 2011 in the hospital B. Twenty nine among 61 strains isolated in the hospital A – belonged to serogroup 3 (Sg3), 28 – were determined as Sg6 and 4 strains – as Sg12. Among 44 strains isolated in the hospital B – 5 different serogrups were determined: *L. pneumophila* Sg1 subgroup OLDA (16 strains), Sg3 (17), Sg 6 (9), Sg12 (1) and Sg13 (1). Strains belonged to Sg3 and Sg6 were found in all water collections in both hospitals. Moreover, the persistent presence of strains Sg1 subgroup OLDA in water distribution system of the hospital B was observed (Fig. 4).

Table 2. Number of *Legionella* spp. in hot water samples collected in the hospital B, in 2004-2011.

Data test D/M/Y	Number of tested samples	Number of *Legionella*-positive samples	Number of *Legionella* spp. [cfu /100 ml] (min –max)	Serogroup	Temperature of water [°C]	Contamination assessment
27.05.04	5	5	$2.0\times10^2 – 4.1\times10^3$	1, 2–14	39–48	medium
07.07.04	5	5	$4.0\times10^2 – 1.9\times10^4$	2–14	29–45	high/very high
10.04.08	4	4	$1.2\times10^2 – 2.3\times10^3$	2–14	42–55	medium
06.07.09	3	3	$3.0\times10^2 – 5.0\times10^2$	nt	nt	medium
Chlorine dioxide disinfection						
17.09.09	3	3	60–80	nt	nt	low
27.05.10	3	3	8–180	nt	nt	low/ medium
A failure chlorine dioxide generator						
10.02.11	4	4	$5.0\times10^2 – 1.2\times10^3$	1, 2-14	nt	medium

nt – not tested

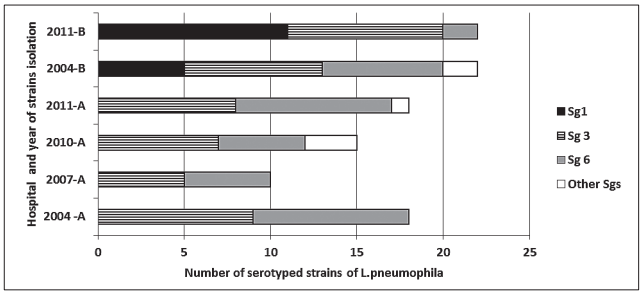

Figure 4. Determined serogroups of *L. pneumophila* isolated from two hot water systems (HWS) in the hospital A (A) and the hospital B (B) by year of water samples collections.

Distribution of strains belonging to particular serogroups in both hospitals HWS was analysed. Almost all water samples collected in the hospital A were positive for strains Sg3 and Sg6 in years 2004 and 2007. This proportion was lower in the next years (2010–2011) but still exceed 50% of collected water samples. In the hospital B, the proportion of water samples positive for *L. pneumophila* Sg1 subgroup OLDA strains was higher after 7 years (44% in 2004y. vs. 75% in 2011y.) (Fig. 5).

Genotyping (sequence based typing of 7 genes) was done for 53 strains, among them 30 isolated from HWS of the hospital A and 23 strains – from the hospital B. Finally, strains belonging to four sequence types (ST) previously described and reported to the EWGLI SBT base were found. There were: ST1 (7 strains), ST81 (3), ST87 (10) and ST114 (14). Moreover, the new STs were reported to the EWGLI SBT base and accepted by EWGLI. There were sequence types: ST835 (6 isolates), ST838 (6), ST956 (1) and ST992 (2 strains).

It was found that the genetic variation was higher than antigenic properties of *L. pneumophila* strains isolated from the both hospitals A and B (Fig. 6). Usually, strains

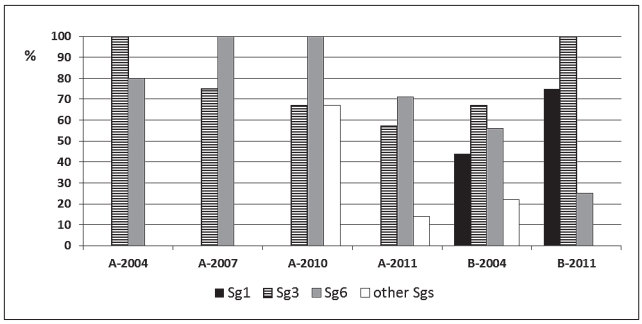

Figure 5. Occurrence of *L. pneumophila* Sg1, Sg3 and Sg6 in HWSs in the hospital A and B – percentage of water samples in which such serogroups were detected.

belonging to the same sequence type, except ST835, show the same antigenic properties. This relation was observed independently on the source of strains (the hospital A or B). In opposite, among strains belonging to Sg3 or Sg6 different genetic types were determined. During serogrouping of strains ST835 isolated in 2004 very strong reactions with monoclonal antibodies against Sg6 were observed. However stronger reactions for MAbs against Sg12 than Sg6 were found when strains ST835 isolated in years 2010–2011 were tested simultaneously.

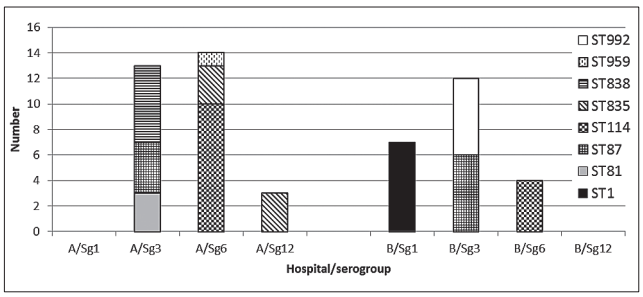

Figure 6. Determined sequence based types (ST) by serogroup of examined strains isolated from HWS of the hospital A and B.

Analysis of SBT results of strains isolated in 2004 and 2011 indicated the occurrence exactly the same ST/strains in HWS of the hospital B after 7 years (Fig. 7). Although the distribution of sequence types of *L. pneumophila* strains isolated from HWS of the hospital A in 2004 and 2011 were similar but not the same. During 7 years of our study some changes of genotypes of isolated *L. pneumophila* strains were

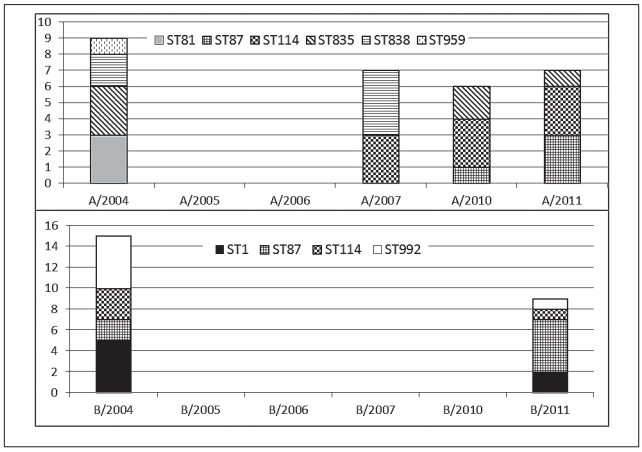

Figure 7. Dynamics of genetic properties of *L. pneumophila* strains isolated in years 2004-2011 in two hospitals: A and B.

observed. Strains belonged to ST81 and ST959 were only found in 2004, strains ST838 – also in 2007. Moreover, strains ST114 have appeared in 2007, ST87 – in 2010. and since then there has been still presented in 2011. Only *L. pneumophila* ST835 has been found during 7 years in the hospital A but their antigenic properties varied as it was described.

Distribution of coding toxin RTX gene was not very high. This locus was found in 7/58 tested strains of *L. pneumophila*, among them one strain isolated in the hospital A in 2004 (*L. pneumophila* Sg6, ST959) and 6 isolates from the HWS of the hospital B in 2004 and 2011. There were 5 strains of *L. pneumophila* Sg1, OLDA, ST1 (71% of tested) and one strain of *L. pneumophila* Sg6, ST114. No one strain among isolates belonging to Sg3 harboured the gene *rtxA*.

DISCUSSION

Contamination of water plumbing system in hospitals is a considerable problem due to the presence of the immunocompromised patients who are hospitalized there (temporarily or permanently). The problem of the *Legionella* spp. elimination from these systems meets difficulties. Both investigated hospitals were built in recent decades (the hospital A – in 1970s, the hospital B – in 1930s). Their water systems were repeatedly rebuilt, new technologies and materials were implemented. Our studies revealed the *L. pneumophila* colonization in both water systems. Both facilities were subjected to chemical disinfection by chlorine dioxide, while the hospital A was subjected to the thermal disinfection also. The lack of expected elimination or ineffective reduction of water systems contamination with the *Legionella* bacteria were undoubtedly the result of inappropriate water systems structures and presence of scale and sludge. Impossibility to keep a strict temperature regime was the crucial factor leading to the *Legionella* bacteria occurrence and proliferation in both cold and hot water. In the majority of cases, the temperature of hot water samples did not exceed 50°C (should be >55°C), and the temperature of cold water samples was higher as usual and reached levels of between 14°C and 31°C (should be <20°C). Results from the temperature of hot as well as cold water indicate that there are serious problems with design of the water distribution systems in both hospitals: too long water pipes cause a considerable heat loss in the case of HWS and leads to the cold water warming up; problems with (or lack of) isolation of cold and hot water pipes/tanks occur and as well as stagnant water in some areas. In such water distribution systems the heat disinfection could not be effective, and could not lead to the elimination of the *Legionella* spp. The problem of construction and design of the water distribution systems may have an impact on the chemical disinfection. The low dose of ClO_2 (up to 0.3 mg/l) caused conditions to worsen *Legionella* spp. proliferation, however didn't considerably reduce the numbers of those bacteria. Only after the dose of chlorine dioxide was significantly changed to the level, when the sum of chlorite and chlorate concentration in point of use was nearly 0.7 mg/l, the considerable reduction of *Legionella* spp. concentration in the hospitals A and B was observed.

However, the implementation of the different methods of water disinfection (including thermal shock) affected the *L. pneumophila* population settled in the hot water

distribution system in the hospital A. It seems that the observed changes in the variants of *L. pneumophila* strains might be explained by using many different methods of disinfections in the hospital A (thermal shock, increase temperature of water and finally continuous ClO_2). Some strains were still occurred after 7 years (ST835 Sg6), some modified antigenic properties (ST835 – Sg12 vs. Sg6), some were eliminated or maybe not detected (ST81, ST838, ST959). This dynamics in *L. pneumophila* population observed during 7 years might be the result of appearance/absence of particular strains or changes in the predominance of these strains. It maybe also caused by random choice of bacterial strain to serotyping and after it – to genotyping.

In the hospital B, the water was treated only with the continuous chemical disinfection which considerably reduced level of that bacteria, but did not cause changes in the *L. pneumophila* population during 7 years of the study. As a result of the breakdown, exactly the same strains of *L. pneumophila* proliferated again to the level which threatened the patients' health. We can obviously presume that there are a lot of different *Legionella* spp. species in the hospital's water system. We should take into account the fact that these species may temporarily increase their numbers or can be detected accidentally.

A legal obligation to register the legionellosis disease in Poland was introduced in 2002, and its causative agent *L. pneumophila* can be found on the list of pathogenic agents within the scope of provisions of the Act on prevention and control of infectious diseases of December 5th, 2008 [20]. In Poland since January 1st, 2008, according to the Ordinance of the Minister of Health from March 29th, 2007 [14], bacteria *Legionella* should be tested in hot water in closed health care buildings. An acceptable value limit for *Legionella* spp. concentrations in hot water cannot exceed 100cfu/100ml. However, we should notice that according to that ordinance, in closed health care buildings in the wards where immunocompromised patients (including patients being subject to immunosuppressive treatment) are hospitalized, *Legionella* spp. should not be present in the water samples of 1000 ml. From the figures presented in the Sanitary State Inspection's data it is apparent that in 2008 and 2009 the sanitary services examined hot water samples from accordingly 72.4% (687/949) and 67.8% (661/975) of facilities which were under their supervision. An acceptable number of *Legionella* spp. (100 cfu/100ml) was exceeded in 2008 in 77.3% and in 2009 in 82.7% of controlled hospitals. Based on data in literature we can clearly see that contamination of water distribution systems in closed health care buildings, also in other countries, often exceeds 50% of examined facilities, and number of detected bacteria of *Legionella* above 10^3 cfu/100 ml might be a real threat for the patient's health [1, 4, 13, 21].

Hospital acquired infections represent 6–7% of all reported annually to ELDSNet cases of legionellosis. Even if the number of reported nosocomial cases of *L.* pneumonia (HAP-LD) is lower than the travel associated legionellosis (TAP-LD, ~20%) or community acquired LD (CAP-LD, > 50%), however the mortality is much higher (up to 50% vs. 3–20%) [12]. The low proportion of HAP-LD may be also connected to problem of diagnosis of legionellosis caused by *Legionella* spp. other than *L. pneumophila* or even caused by *L. pneumophila* non-Sg1. Some diagnostics tests are focused on *L. pneumophila* Sg 1 only/mainly [22, 23]. As it was described by Helbig et al. [16] the ratio of other than *L. pneumophila* Sg1 among HAP cases was significantly higher in the Central Europe as well as in the Scandinavian countries than in the Mediterranean region.

However, we should take into the consideration the fact that the risk assessment based only on technical parameters (water temperature, ClO_2 concentration, material used in construction of water distribution system etc.), extent of colonization (percentage of *Legionella* spp. positive samples), contamination level (number of bacteria) may not provide a full picture of the risks. There were some legionellosis cases encountered in a few hospitals in spite of the fact that the number of *Legionella* in water was below 100 cfu/100, and the risk assessment indicated that the health risk is not serious. Complex risk assessment should be supplemented with the additional examination like *L. pneumophila* serological typing (at least serogrouping), genotyping and virulence testing of isolated strains.

As it was shown in our studies, both hospitals (A and B) were colonized by *L.pneumophila* strains. However, some difference were found. Our studies indicated that in the hospital A, only after application of maximum dose of chlorine dioxide, the number of *Legionella* spp. was considerably reduced. Moreover, the identified antigenic and genetic properties of isolated strains indicated that the health risk in that hospital is not serious. However, the time of exposure should be also taken into consideration. In such a hospital, where highly specialized medical procedures are done, the period of hospitalization might be longer, so the risk of legionellosis for selected patients might be higher than it was estimated basing on the proportion of positive samples, number of *Legionella*, determined serogroups and sequence types of isolated strains. The longer patients are hospitalized and the more their immune systems become suppressed, the more serious the risk becomes. Nevertheless, testing of the water samples are recommended.

In the hospital B, the persistent colonisation with the same strains of *L. pneumophila* was observed after 7 years. There were identified strains belonging to 4 sequence types, and one of them (ST1) indicated the link with hospital acquired pneumonia (according to the EWGLI SBT base) [18]. Moreover, based on the results of the additional examinations (serotyping, RTX), it was concluded that this strain *L. pneumophila* Sg 1, subgroup OLDA, ST1, *rtx*A+ showed high virulence properties [21]. Bacteria belonging to this strain were found in 75% of water samples collected in 2011y. and gene for RTX toxin was harboured by the majority of them. The ability of *Legionella* to enter and replicate in monocytes is essential for pathogenesis. RTX proteins are involved in a bacterial surface-associated cytotoxic activity and this play role in adherence, pore formation, intracellular replication, and virulence of *L.pneumophila* [19].

All of the obtained data indicated that in the hospital B the risk of nosocomial legionellosis is high and much higher than in the hospital A. In such a situation, the information on probability of nosocomial legionellosis should be communicated to clinicians for the appropriate treatment in a suspicion of legionellosis. Also the hospital management should be informed in order to take an appropriate action. Moreover, the hospital laboratory should have possibility to diagnose and confirm *Legionella* infection due to this strain. According to our results, the diagnostics system of *Legionella* infections in this hospital at least should consist of a test for detection of *L. pneumophila* antigen in urine

samples. This fast test done in the first step of LD, which confirm the legionellosis by positive result. However, it must be pointed out that not all commercial tests for diagnosis of *L. pneumophila*, even focused on *L.pneumophila* Sg1, allow to identify the infection due to *L. pneumophila* Sg1, subgroup OLDA [22, 23, 24]. The selection of diagnostics tests should allow to identify also cases due to *L. pneumophila* non-Sg1, so PCR, culturing or serological assays should be also available.

It must be pointed out that determination of number of *Legionella* spp. in water sample by culturing is the recommended method according to the MH Ordinance. This is the "gold standard" but it takes at least 10–14 days. Nowadays, a lot of new tests/methods were developed for faster, cheaper and adequate detection of *Legionella* spp. in water samples. The main problem of examinations by PCR technique is that this method allows to detect DNA of viable and dead cells together. The selection of primers and determination of genes expression may be a solution. Moreover classical PCR is qualitative method so only Real-Time PCR may be used for quantitative analysis. Also in Poland studies on use Real-Time PCR for detection and determination of number of *Legionella* spp. in water samples are done [25].

SUMMARY

In accordance to the World Health Organization (WHO) and European Legionnaires' Disease Surveillance Network (ELDSNet) recommendations, all artificial water systems, where *Legionella* bacteria can appear and proliferate, should be systematically monitored [26]. Additionally, Polish law regulations – the Ordinance of the Health Minister and the Ordinance of the Minister of Infrastructure point out the necessity of monitoring the hot water systems. Our studies, as well as others projects indicate that in the hot and cold water systems of hospitals, occur favourable conditions to *Legionella* bacteria occurrence and proliferation. A large number of isolated bacteria cause a real risk of legionellosis for hospitalized patients in closed health care buildings, including medical staff. It is necessary to implement the system of monitoring the quality of water and implement a schedule of interim review of internal water distribution system and other devices generating aerosol of airborne water droplets. Compliance with these legal requirements, including maintenance of the sanitary regime and monitoring of risk factors should reduce the health risk to patients [1, 21, 26].

Differentiation and typing of environmental *Legionella* spp. strains, even identification the species *L. pneumophila*, is very expensive. However, on the basis of obtained results, we were able to demonstrate the persistent colonization with strains of *L. pneumophila* belonging to ST114 and ST87 of the HWS in the hospital A, and in the hospital B – by strains ST1, ST87, ST114 and ST992. The information on persistent colonization with these strains is very important for risk assessment of legionellosis in both hospitals. Analysis based on our results (genotype, serogroup, proportion of *Legionella* positive water samples, number of *Legionella* spp.) and data in the EWGLI SBT base indicated the highest (in our study) risk of *Legionella* infection due to strains *L. pneumophila* Sgp1, subgroup OLDA, ST1, *rtxA*+ colonized of the hot water system in the hospital B. Such strains (*L. pneumophila* Sgp1, ST1) were often isolated during epidemiological investigation in case of nosocomial *Legionella* pneumonia from both materials: clinical and environmental [18, 21]. One of the latest fatal case of infection due to *L. pneumophila* Sg1, subgroup OLDA/Oxford, ST1 was reported in Israel (February 2012) in an infant under 6 months of age [27]. It was the case of community acquired pneumonia but *L.pneumophila* ST1 strains are frequently related to nosocomial infections (>50% the strains in the EWGLI SBT base).

Genotyping, serogrouping and subgrouping, detection of *rtxA* gene and other virulence markers are very expensive but the obtained data are very informative (in analysis with the SBT EWGLI base) and might be very useful in an epidemiological investigation or a complex risk assessment. Such complex and very expensive analysis should be done in substantiated cases (as it was mentioned) by reference laboratories. The ability to perform such complex tests is necessary for the appropriate diagnosis of cases, identification of outbreak and quick and adequate reaction – as it was shown this year in Edinburg, Scotland [28]. In Poland, the lack of such systems is still observed.

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