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ORIGINAL ARTICLE

Sequence-based typing of *Legionella pneumophila* strains isolated from hospital water distribution systems as a complementary element of risk assessment of legionellosis in Poland

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Abstract

Many factors affect the risk of Legionella infection, such as the design, construction and maintenance of water distribution systems, the presence of individuals who may be exposed and their vulnerability to infection, and the degree of water system colonization and properties of Legionella strains. For epidemiological investigations, two properties of the Legionella strains are usually determined: serotyping and genotyping (sequence-based typing, SBT). In Poland, data regarding legionellosis are fragmentary, despite the fact that this has been a notifiable disease since 2002. The number of reported cases is very low; moreover, the main method of diagnosis is serological examination (delayed diagnosis and cheaper methods), and only single cases of LD were confirmed by culture of bacteria. Therefore, after 10 years of mandatory reporting of the Legionella spp. infection in Poland, the real epidemiological situation is still unknown; however, risk assessment should be carried out, especially in hospitals. In the presented study, comparison of the sequence types of 111 isolated L. pneumophila strains (from hospital water systems) with those present in the EWGLI SBT data was undertaken for complex risk analysis as a complementary element. In total, strains of L. pneumophila belonging to 12 out of 19 STs determined in the presented study were previously reported to the EWGLI SBT database (ST1, ST42, ST59, ST81, ST87, ST114, ST152, ST191, ST371, ST421, ST461, ST520). Among these strains, only 7 STs were previously reported in the amount of ≥10 (mainly ST1, ST42, ST81). Analysis of EWGLI data were carried out and, proportionally, the highest percentage of hospital-acquired strains (clinical and environmental) was found for ST 81, ST421 and ST152, but the largest number was for ST1. Based on the EWGLI data and the presented results, it was found that persistent colonization of HWS of 3 hospitals by strains belonging to ST42, ST1, ST87 indicated an increased risk of legionellosis, especially ST42.

Key words

Legionellosis – prevention & control, risk assessment, sequence-based typing, the EWGLI SBT database

INTRODUCTION

The *Legionella* bacteria are Gram-negative rods common in environmental water sources (rivers, lakes, soil) and artificial reservoirs (e.g. water distribution systems, cooling towers). The detected number of *Legionella* bacteria in artificial reservoirs might be higher than in the natural environmental sources because of the favorable conditions for *Legionellae* growth, such as temperature within the 20 °C – 60 °C range and the presence of nutrients (sediment, scale, sludge and biofilm). The *Legionella* bacteria, especially the *L. pneumophila,* are an aetiological agent of legionellosis. The most common forms of legionellosis are pneumonia (Legionnaires' disease, LD) and flu-like infection (e.g. Pontiac fever). It is estimated that 5–15% of community-acquired pneumonia in Europe may be caused by *Legionella* spp. [1, 2, 3].

In Poland, legionellosis has been a notifiable disease since 2002. Unfortunately, the number of reported cases is still very low, which is probably because clinicians and GPs rarely suspect this disease. The number of clinical specimens tested for the *Legionella* spp. infection is very low, and the majority of them are collected in the late phase of the disease. Moreover, in Poland, the main method of *Legionella* infection diagnosis is serological examination (delayed diagnosis and cheaper methods). Thus, after 10 years of mandatory reporting of the *Legionella* spp. infection in Poland, the real epidemiological situation is still unknown [4]; however, risk assessment should be undertaken.

The risk assessment of legionellosis is a very complex task. Many factors affect the risk of *Legionella* infection, such as the design, construction and maintenance of the water distribution system; the presence of individuals who may be exposed and their vulnerability to infection; the degree of water system colonization (number of *Legionella* spp. cfu/L; the percentage of *Legionella* spp. positive samples) and properties of *Legionella* strains. Pathogenicity of *Legionellae* for humans depends on many different factors which are involved in the ability to invade and grow into cells, as well as to evade from human cells. Nowadays, many different mechanisms of pathogenicity are studied [5, 6, 7, 8, 9, 10, 11]. However, for epidemiological investigations, two properties of the *Legionella* strains are determined: the serological group (especially *L. pneumophila* sg 1) and the genetic type. Sequence-based typing (SBT) is considered as the 'golden standard' of genotyping [12, 13, 14, 15, 16].

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OBJECTIVE

The aim of the presented study was comparison of the sequence types of 111 isolated *L. pneumophila* strains (from hospital water systems) with those present in the EWGLI SBT data, carried out for complex risk analysis as a complementary element.

MATERIAL AND METHODS

Isolation and identification of bacterial strains. Isolation and identification of *Legionella* spp. was performed in compliance with the ISO 11731 method (ISO 1998). Briefly, after concentration of a water sample, filters were inoculated on a plate with GVPC and incubated at 36+/-2 °C for 10 days. Colonies which were visible after 2 or more days, were tested for cysteine auxotrophy. The *Legionella* colonies were confirmed and typed with specific antibody latex agglutination reagents (Oxoid). This test allows determination of *L. pneumophila* belonging to sg 1, and to sgs 2–14 and *Legionella* spp. strains. More detailed serotyping examinations were carried out using the Dresden MAb Panel, kindly provided by Dr Jurgen Helbig from the Institute of Medical Microbiology and Hygiene at the Medical Faculty of the Technical University in 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 monoclonal antibodies (MAb).

Selection of *L. pneumophila* strains for SBT. For the purpose of genotyping, strains of *L. pneumophila* were selected. The criteria of selection were place of isolation of the *L. pneumophila* strains and their antigenic properties (determined serological groups and subgroups). The number of *L. pneumophila* strains selected for genotyping belonging to particular serogroups and subgroups was determined, based on the proportion of serogroups/subgroups of all tested isolates from this specific water distribution system. This rule was used in every tested water distribution system.

The investigation included 109 *L. pneumophila* strains isolated from the water distribution system in 9 hospitals in Poland. Eight of the hospitals (coded A-H) were located in Warsaw, and one hospital (code J) located outside Warsaw. Water samples were collected during the period 2001–2011 as part of research projects 2P05D 026 26; 2004–2007 and NN 404 099536; 2009–2011, or routine examinations of the hot water systems (HWS). Three hospitals were examined more than once (3–4 times). Moreover, 2 *L. pneumophila* strains isolated from the HWS of one hospital (code K) in Kraków were also included in this study, making a total of 111 strains (Fig. 1).

STRAIN GENOTYPING.

DNA extraction, PCR amplification. The strains **s**tored 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, Poland) using GoTaq Flexi DNA polymerase (Promega, Madison, USA).

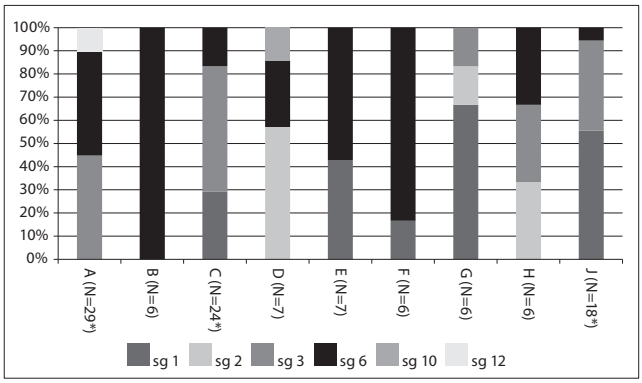

Figure 1. Percentage and number of *L. pneumophila* strains selected for SBT, by serogroup and hospital.

*- HWS tested more than once; N= number of genotyped *L. pneumophila* strains.

SBT. Genotyping was performed according to the 7-gene protocol from the EWGLI SBT scheme (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/ php/sbt_homepage. php). Sequences were analysed with the use of the online tool BLAST (http://blast.ncbi.nlm.nih.gov), or online available Legionella SBT Quality Assessment database (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi). The assignment of the sequence type (ST) was carried out with the use of the SBT database checker (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php). For each isolate, the profile of 7 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.

Statistical analysis. Multivariable analysis, correlations, and sample comparisons were performed with the use of Statgraphics Centurion v.XV.

RESULTS

Totally, among the tested 111 isolates of *L. pneumophila* (belonging to 6 different serogroups), 19 different sequence types (ST) were found (Fig. 2). For 5 strains, no STs were determined because of the lack of one out of 7 amplicons.

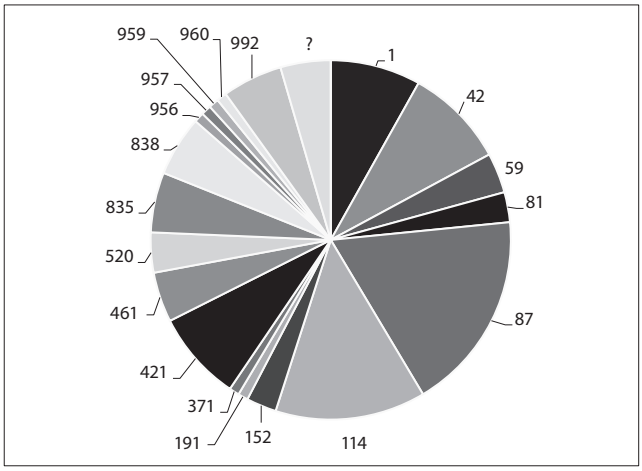

Figure 2. Determined sequence types (ST) of 111 *L. pneumophila* strains.
? – undetermined sequence type; 42 – sequence type 42 (ST42).

Table 1. Sequence types, allelic profiles and serogroups of 111 *L. pneumophila* strains isolated from 10 Polish hospitals (HWS). New profiles are in bold characters.

ST	Alleles profile	Sg	Subgroup	No. of tested isolates	Hospitals (year of water sample isolation)
1	1;4;3;1;1;1;1	1	OLDA	9	C (2004,2011); K (2006)
42	4;7;11;3;11;12;9	1	Benidorm	10	J (2006,2007)
59	7;6;17;3;13;11;11	1	Camperdown	4	G (2005)
152	1;4;3;1;1;1;3	1	OLDA/Oxford	3	E (2001); F (2005)
960	**2;6;3;6;1;4;9**	1	Philadelphia	1	E (2001)
81	2;10;3;28;9;4;9	3	-	3	A (2004)
87	2;10;3;28;9;4;13	3	-	20	A (2010,2011); C(2004,2011); J (2006); H (2011);
114	3;6;1;6;14;11;9	6	-	15	A (2011,2010,2007); C (2004,2011); D (2004)
191	6;10;19;28;19;4;6	6	-	1	B (2004)
371	2;10;17;28;9;4;13	3	-	1	G (2005)
421	2;10;3;3;9;4;3	6	-	9	B (2004); E (2001)
461	6;10;14;28;21;14;9	6	-	5	F (2005); H (2011)
520	2;10;24;28;4;4;13	2	-	4	D (2004)
835	**2;6;3;6;9;4;9**	6 12/6	-	3 3	A (2004); A (2010,2011)
838	**3;6;1;28;14;11;9**	3	-	6	A (2004,2007)
956	**6;10;3;3;9;1;9**	6	-	1	F (2005)
957	**7;6;17;3;21;11;9**	6	-	1	J (2006)
959	**1;4;3;1;9;13**	6	-	1	A (2004)
992	**3;6;1;28;14;11;13**	3	-	6	C (2004,2011)
?	2;10;24;28;4;4;0 6;10;1;3;0;4;9 6;10;3;12;9;4;0	2 6 10	- - -	3 1 1	G (2005); H (2011) F (2005) D (2004)

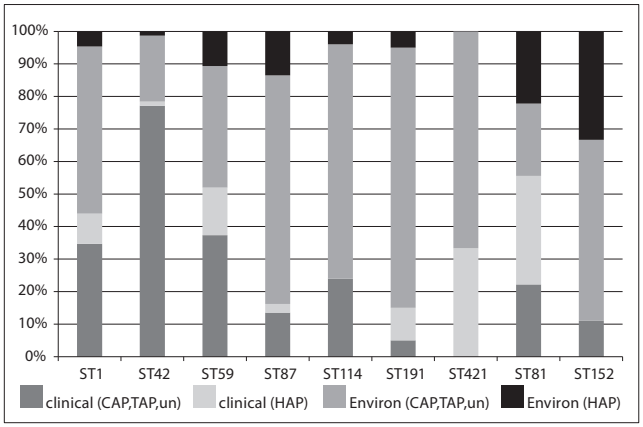

Figure 3. EWGLI SBT database – distribution of the *L. pneumophila* strains involved in hospital infections among the selected 9 sequence types: determined in Poland and reported to the EWGLI SBT database in the number of ≥9.
L. pneumophila strain isolated from a clinical specimen and reported to the EWGLI SBT database;
Environ – *L. pneumophila* strain isolated from an environment and reported to the EWGLI SBT database;
CAP – community-acquired pneumonia;
TAP – travel associated pneumonia;
HAP – hospital acquired pneumonia;
un – unknown category of pneumonia due to *Legionella* spp. (CAP or TAP or HAP).

The product of the *neuA* gene amplification was missing in the examination of 3 strains belonging to sg 2 (isolated in 2 different hospitals), and one strain – sg 10 (isolated from the third hospital). However, 3 different profiles of 6 alleles were found. The product of the *momp*S gene amplification was insufficient for the sequencing of one strain belonging to sg 6, isolated from hospital F.

Twenty-seven isolates of *L. pneumophila* sg 1 strains divided in 5 subgroups (Philadelphia, Benidorm, OLDA, Oxford, Camperdown) were examined. Five different STs were determined: ST1, ST42, ST59, ST152 and ST960 – a new ST. The tested strains belonging to the same sequence type (ST) had the same antigenic properties (Tab. 1).

Strains of *L. pneumophila* sg 6 (37 strains; 33.3% tested) were found in the water distribution systems in almost all hospitals (8/9 hospitals). Among the 37 strains, 9 different allelic profiles were determined. Four profiles were previously described and reported but 4 profiles were new. The highest distribution/occurrence was found for ST 114 (15 strains found in 3 hospitals). Moreover, 3 ST 835 strains were determined as sg 12, but strong cross-reaction with mono-antibodies for sg 6 was observed.

Strains of *L. pneumophila* sg 3 (36 strains, 32.4% of all tested strains) were found in the water distribution systems of 5 hospitals. Five different sequence types were determined, 2 of which were new types. The most common genetic type, ST 87, was found in 4 hospitals.

Among the *L. pneumophila* sg 2 strains (7 strains; 6.3%) 2 different profiles of alleles were found: one completed ST520 (7 alleles) and one not completed – only 6 alleles.

A significant correlation between the determined sequence type of the tested *L. pneumophila* strains and their antigenic properties (serogroups) was found (P value= 0.0000). The analyzed 111 strains belonged to one sequence type and had the same antigenic properties (with one exception – ST835); however, strains belonging to one serogroup might be a different genetic type. Moreover, a significant relation was also found (P value=0.0000) between ST determined in the presented study and the source of the strains, i.e. the hospital. Those correlations indicated that every system of water distribution should be recognized as a separate ecosystem.

In 3 hospitals, water samples were collected more than once. Some of the *L. pneumophila* strains were found to be persistent: strains belonging to the same serogroups and ST were found in the HWS of hospital C after 7 years (ST1, ST87, ST114, ST992). In this hospital, continuous chemical (chlorine) disinfection was carried out, but the system failed in 2011. Strains of *L. pneumophila* ST 114, ST87 and ST835 seemed to be persistent flora of the hot water system in hospital A, no matter which disinfection action was used: chemical shock (chlorine) or thermal shock. However, some changes in antigenic properties of the *L. pneumophila* strains were observed in this hospital: *L. pneumophila* ST 835 were determined in 2004 as belonging to serogroup 6, but in the next examinations (2010–2011) as sg 6/12.

DISCUSSION

The EWGLI SBT database was created as a faster and easier way for interregional/international communication and the possibility to compare the genotyping results obtained in

different laboratories and countries. In the beginning, the majority of strains reported to the EWGLI SBT database were isolated from clinical specimens or were connected with the cases of Legionnaires' disease. Nowadays, strains isolated from routine water examinations are also reported. Analysis of data collected in the EWGLI SBT database demonstrated huge differentiation in the number and spread of particular sequence types. The most widespread genotype of *L. pneumophila* was ST1 – reported by 27 countries, including Poland, and from all continents (data from 2 February 2012). Strains belonging to ST42, ST23, ST62, ST59 and ST114 were found in the countries of at least 3 continents (Tab. 2). ST1 strains were also the most numerous in the EWGLI SBT database, followed by ST23 and ST47. These strains were reported mostly as being isolated from clinical samples (above 90% reported). Such a high rate of strains isolated from patients with legionellosis was also observed for the genotypes ST 62, ST20 and ST146.

Table 2. The EWGLI SBT database – the selected most common sequence types of *L. pneumophila*

ST	Total number of reported strains	% Clinical strains	Countries reported	Geographic regions/countries where strains were reported
1	761	44%	27	Europe, North and Central America, Japan, China, Africa, Australia
23	410	>90%	17	Western and Southern Europe, Japan, USA
47	410	>90%	10	Western and Southern Europe, Canada
62	174	>90%	15	Western and Southern Europe, USA, Canada, Japan
42	155	77%	25	Europe, America, Asia, Africa
20	83	>90%	10	Western and Southern Europe,
59	76	52%	10	Europa, Russia, North America
146	69	>90%	8	Western Europe, Russia
292	26	<5%	6	Western Europe, Russia
87	37	16%	7	Europe, Russia
114	25	24%	11	Europe, Russia, Canada, Japan, Singapore

Based on the proportion of clinical strains belonging to particular sequence types reported to the EWGLI SBT database, some trends can be observed. There are strains mainly reported as an environmental *L. pneumophila* (% of clinical strains <=30%), strains similarly reported as clinical and environmental (40–60%), strains mainly reported as an aetiological agent of LD (70–80%), and strains very strongly connected with disease (>90%). Moreover, distribution of genotypes of the *L. pneumophila* strains involved with nosocomial infections (both origin of strains: clinical and environmental) varied. Some genotypes were more frequently reported as an agent of hospital-acquired pneumonia than others – more than 20% of clinical strains belonging to ST1 and ST59 were associated with nosocomial legionellosis (Fig. 3). Diversity of the *L. pneumophila* strain properties: genetic, antigenic and virulence, is very high and not yet fully described. However, the *L. pneumophila* strains belonging to some sequence types or serogroup/subgroup were more often described as an etiological agent of pneumonia, than other isolates. There are broad fields for discussion: why these strains were more frequently isolated from patients with LD; why strains belonging to ST1 or ST42 were reported from so many countries; are these strains really so widespread? The answers are still unclear, but some trends can be noticed.

Risk assessment is a multi-element analysis, although the determination of only one feature of isolated *L. pneumophila* strains (sequence type or serogroup) is not sufficient. However, comparison of the sequence types of isolated strains with those present in the EWGLI SBT data, might be very useful in complex risk assessment of legionellosis, as a complementary element, especially when repeated LD cases were observed for some STs. Moreover, this kind of analysis might be useful when typing data are available from environmental strains/data only. Such a situation was observed in Poland where only single cases of LD were confirmed by culture of bacteria.

In Poland, data regarding Legionnaires' diseases and other forms of legionellosis are fragmentary. For this reason, the SBT database of EWGLI was used for a risk analysis.

In total, strains of *L. pneumophila* belonging to 12 out of 19 STs determined in the presented study were previously reported to the EWGLI SBT database (ST1, ST42, ST59, ST81, ST87, ST114, ST152, ST191, ST371, ST421, ST461, ST520). Moreover, *L. pneumophila* ST 956 firstly reported in Poland, was also found in Germany. Six other STs were first reported only in Poland (Tab. 1.).

Among the genotypes (13 STs) of *L. pneumophila* strains isolated in Poland and reported to the EWGLI SBT database, only 7 STs were found in the EWGLI SBT database in the number of ≥ 10. These were sequence types: ST1, ST42, ST59, ST87, ST114, ST191 (20 reported strains), ST421 [12]. Moreover, strains ST 81 and ST152 were reported in the number of 9. The remaining STs determined in the presented study are represented by a small number of strains (<5) in the EWGLI database.

Data regarding strains reported worldwide to the EWGLI SBT database were compared with those found in Poland. The ST most common in the world were strains belonging to ST1; however, the percentage of strains isolated from clinical samples was 44%. Strains of *L. pneumophila* ST1 were reported to the EWGLI SBT database by 27 countries. Strains ST42 were reported in 25 countries, although not in large numbers, but the percentage of clinical strains was very high (77%). The largest number of reported strains was usually connected to the largest number of reporting countries.

Analysis of the percentage of clinical strains isolated from hospital-acquired cases of Legionnaire's disease, or environmental strains connected with nosocomial legionellosis, indicated a high variation between the genotypes. Proportionally, the highest percentage of hospital-acquired strains – both, clinical and environmental – was found for ST 81, ST421 and ST152. However, the largest number of those reported strains was for *L. pneumophila* ST1 – more than 100 strains.

It should be emphasised that ST determination of the *L. pneumophila* strains might be useful for the detection of persistent colonization of HWS. Persistence in hospital HWS of *L. pneumophila* strains belonging to sequence type involved in a larger number of LD cases (% of clinical strains >50%) might increase the risk of legionellosis. Based on this principle, the risk assessment for hospital C, after additional examination of strains isolated from the HWS, should be increased because persistent *L. pneumophila* strain ST1/sg 1/ subgroup OLDA was still observed after 7 years, despite chemical disinfection. A few nosocomial cases of

Legionnaires' disease were diagnosed in this hospital during the past 10 years, based on the date of hospitalisation and results of urinary antigen assays or serological tests, but without culture of *L. pneumophila* strain from clinical samples. The highest importance among examined strains, based on the EWGLI SBT data, was found for strains belonging to ST42 (>70% of clinical isolates). Such a strain was identified in hospital J, where a nosocomial outbreak of LD was described and 3 out of 4 patients died because of LD (urinary antigen+, PCR+, seroconversion). Once again, no *Legionella* strain was cultured (lack of appropriate clinical specimens sent to *Legionella* culture). These two situations described very well the difficulties in an epidemiological investigation in cases of legionellosis in Poland – the diagnosis is usually based on a single examination result, and mainly on the serological test, because of the very belated suggestion of *Legionella* infections posed by doctors. The lack of a *Legionella* reference laboratory is the reason why all tests (for diagnostic and epidemiological purposes) must be paid for by the patients, doctors or hospitals. The very small number of legionellosis suspicions is the cause for the very small number of tested clinical samples, and then by the very small number of diagnosed cases. However, the very small number of suspected *Legionella* infections was caused by the lack of awareness among doctors, health and municipal services of the possibility of such an infection in Poland. This why the risk assessment of *Legionella* infections should be carried outdone, even based on the very limited Polish data, but in conjunction with the international complex data.

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