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Sequence based typing and pre-absorption test in retrospective analysis of a pseudo-outbreak of *Legionella* infections differentiates true cases of legionellosis

Katarzyna Pancer[1], Karina Jahnz-Różyk[2], Aleksandra Kucharczyk[2], Włodzimierz Gut[1], Piotr Kucharczyk[2], Pernille L. Elverdal[3], Bogumiła Litwińska[1]

[1] *National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland*
[2] *Department of Immunology and Clinical Allergology, Military Institute of Medicine, Warsaw, Poland*
[3] *Serum Production, SSI Diagnostica, Statens Serum Institute, Hillerød, Denmark*

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Abstract

The aim of this study was elimination of false positive results obtained by the Chlamylege kit. Two serological kits (IgM ELISA L.pneumophila sgs1-7; ImmuView™ L.pneumophila sg1/sg3) and pre-absorption tests (with L.pneumophila sg1 and sg3 reference strains antigens) were used. 153 sera (79 patients) were examined. The high correlations were found between the results by both tests. Positive results by ELISA (sgs1-7) were found in 19/79 patients; by ImmuView™ (sg1+sg3) in 16/63. In 8 patients, the dynamics of the IgM in pairs of sera was high (ratio ≥2). In 5/8 of those patients seroconversion was determined. Selected pre-absorbed sera (15 pairs) were tested simultaneously by the same tests. In 8/15 pairs of sera, the reduction of IgM levels in pre-absorbed sera was higher than 10. The reduction of IgM differed in sg1 and sg3 tests. The probability of infections due to L.pneumophila sg3 (7 patients) and L.pneumophila sg1 (5 patients) was based on the results of pre-absorption tests. The correlation between ELISA and ImmuView™ tests of pre-absorbed sera was statistically significant (Po=0.0389). Moreover, genotyping of L.pneumophila (SBT) directly in the sera of selected 15 patients (high IgM reduction) was carried out. Completed 7 alleles profile (ST36) was determined in one patient. However, a second patient had the same profile of 5 alleles, and similar reactions in pre-absorption tests. At least 4 sources of infections were suggested on the base of genotyping and pre-absorption results.

Conclusions: Positive results obtained by molecular techniques (eg.PCR) in the diagnosis of *Legionella* infections should be supplemented by other tests for confirmation of legionellosis. The sequence based typing carried out directly in clinical specimens seems to be a promising method.

Key words

legionellosis, pseudo-outbreak, false positive results, *Legionella* serology, pre-absorption test, sequence base typing.

INTRODUCTION

Two different forms of legionellosis – a disease caused by the bacterium *Legionella spp.* – can be observed: pneumonia (Legionnaires' disease, LD) and extrapulmonary diseases (non-pneumonia). Among non-pneumonia legionellosis, the form most often described is Pontiac Fever (PF) [1, 2]. *Legionella* pneumonia are mandatorily notifiable in most European countries. Currently, 35 countries cooperate within The European Working Group for *Legionella* Infections (EWGLI), established in 1986 for the surveillance of *Legionella* pneumonia cases, especially related to travel within and outside Europe. In Poland, legionellosis has been mandatorily notifiable since 1st January 2002, but as opposed to EWGLI, both forms of legionellosis (pneumonia and non-pneumonia) should be investigated and notified. The number of reported cases of legionellosis in Poland is still very low: 210 cases of legionellosis were reported during the years 2003-2010 [3]. In comparison, the total number of *Legionella* pneumonia cases reported to ELDSNet/EWGLI in 2010 was 6,150 (preliminary data). According to EWGLI data, 50-70% of all annually reported cases of LD should be categorized as community acquired *Legionella* pneumonia (CALP), 20-25% as travel associated *Legionella* pneumonia (TALP), and 5-10% as hospital acquired *Legionella* pneumonia (HALP) [1, 4, 5]. In most cases of *Legionella* infections reported in Poland, both the source of infection and the category of disease was unknown.

The introduction of diagnostic methods for the detection of legionellosis in hospital laboratories meant an increase in the number of detected infections, usually. The reason for this may be the research conducted in the Department of Immunology and Clinical Allergology at the Military Institute

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

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of Medicine in Warsaw. In the period July 2009 – April 2010, diagnostics for atypical pneumonia agents was introduced. Two-stage diagnostics, based on molecular methods – PCR and hybridisation, were made using the Chlamylege test for detecting *Chlamydia pneumoniae, Mycoplasma pneumoniae* and *Legionella* species (spp.)/*Legionella (L.) pneumophila*. An unexpectedly high incidence of positive results obtained for *Legionella* spp./*L. pneumophila* raised concerns, and led to the need to undertake additional, retrospective studies to confirm *Legionella* spp. infections.

The aim of the presented study was to eliminate false positive results obtained by molecular methods. For this purpose, 2 different serological commercial kits and pre-absorption tests (with antigens of reference *L. pneumophila* strains) were used in order to exclude cross-reaction, and to confirm *L. pneumophila* serogroup (sg) 1 or sg 3 infections. The next aim was to determine by genotyping technique based on sequencing (SBT) whether the confirmed cases of *Legionella* were sporadic infections, or part of a cluster/outbreak.

MATERIALS AND METHODS

The study was conducted within a research project, the main objective of which was to determine the frequency of infections caused by viral agents (*Respiratory Syncytial Virus*, RSV and *human Metapneumovirus*, hMPV) and/or bacterial atypical agents (*Mycoplasma pneumoniae* and *Chlamydia pneumoniae*) in patients with chronic respiratory diseases. The studies were undertaken during the period October 2008 – March 2011. All patients were informed about the project (subject, scientific targets, and limits) and agreed to participate in this study, (according to the decision of The Bioethics Committee at the Military Institute of Medicine, No. 87/WIM/2006).

Characteristics of patients. The selection of patients was made by clinicians based on the clinical symptoms. Diagnostics was performed on patients visiting the Department of Immunology and Clinical Allergology; the inclusion criteria were respiratory tract infections, exacerbations of asthma or chronic obstructive pulmonary disease (COPD). A total of 94 patients were included in the primary project. At first diagnoses of infections caused by *Legionella* were not included in this project, but the use of the Chlamylege kit for detecting *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella* spp. (Argene S.A., USA) allowed studies of infection with *Legionella* spp. In total, 108 respiratory specimens were tested by the Chlamylege kit for *L. pneumophila* in the period January 2009 – October 2010 in the hospital. The number of Chlamylege/*L. pneumophila* positive samples increased slowly in June 2009 and sharply increased in September 2009. On the base of PCR results, 3 periods were determined:

1. January – 8 June 2009: no *L. pneumophila* positive samples;
2. 8 June 2009 – 28 April 2010: 93% *L. pneumophila* positive samples;
3. 29 April 2010 – 31 October 2010: 24.3% *L. pneumophila* positive samples of tested respiratory samples.

At the end of April 2010, new reagents and new filters were used and the number of positive Chlamylege results decreased in the third period. There was a significant relationship between the Chlamylege results and the week of respiratory samples collection (Po=0.0001). No other methods were used for detecting *Legionella* infections in this hospital.

In our retrospective study, serum samples were obtained from 79 patients who visited their doctors in the period from January 2009 – January 2011. Fifty-two out of 79 patients in the selected period had been previously examined by Chlamylege for the presence of *L. pneumophila* in their respiratory samples.

The 79 patients were divided into 4 groups, taking into account the major diseases: pneumonia, COPD, exacerbation of asthma, and upper respiratory tract infections (URTI), e.g. persistent cough (Tab. 1).

Table 1. Characteristics of examined patients

Disease	No. of patients	Male	Female	Age	Patients with positive Chlamylege test in respiratory sample/No. of examined patients
Pneumonia	31	19	12	22-84	18/23
COPD	18	11	7	30-82	6/13
Exacerbation of asthma	15	5	10	33-87	3/6
URTI	15	3	12	21-56	4/10

Serum samples. One hundred and fifty-three serum samples from 79 patients were collected. From 29 patients, only one serum sample was obtained, from 27 patients – 2 samples, from 22 patients – 3 samples, and from one patient – 4 samples were obtained.

Serological examinations. Serum samples were tested by Anti-*Legionella pneumophila* sgs 1-7 ELISA (IgM) (Euroimmun, Medizinische Labordiagnostika AG, Lübeck, Germany) according to the manufacturer's instructions (in our laboratory, this test was implemented in the routine diagnostics of legionellosis in 2006); and ImmuView™ *Legionella* Blood Test (Statens Serum Institut, Denmark) for the detection of IgM antibodies against *L. pneumophila* sg 1 and *L. pneumophila* sg 3. This immune-chromatographic quick test was performed according to the manufacturer's guidelines.

Pre-absorption of serum samples with *Legionella* antigens.

- 15 selected serum samples (on the basis of ELISA IgM and ImmuView™ test results) were absorbed with antigen *L. pneumophila* sg 1 reference strain ATCC 33152 (Lp sg 1) and *L. pneumophila* sg 3 reference strain ATCC 33155 (Lp sg 3).
- **Preparation of antigens:** Antigens were prepared according to the procedures for the Micro Agglutination Test (MAT). Briefly: reference strains stored at -70°C were cultured on BCYEα medium. After 2 days of incubation (temperature 37°C, 2.5% CO_2), growth was observed and bacteria were harboured, boiled for 10 minutes and washed 3 times in PBS. The density of the solution was determined. For the absorption, the solution 0.5 McFarland was used.
- **Absorption of serum samples**: A modified method for pre-absorption [6, 7] was used; modifications were developed and validated. The reduction of cross-reaction and increase in the specificity of the reaction in pre-

absorbed sera was determined by using antigens of *B. pertussis*, *L. pneumophila* sg 1 and *L. pneumophila* sg 12. Pre-absorbed sera were previously positive for *B. pertussis*, *L. pneumophila* sgs 1-7 (by ELISA) *or L. pneumophila* sg 1 *and L. pneumophila* sg 12 (by MAT), or for more than one aetiological agent. Moreover, the method of pre-absorption (with antigens of *L. pneumophila* sg 1 or sg 3 or both antigens) was validated by examination of serum samples collected from patients with confirmed Legionnaire's disease with at least 2 different methods (culture, urinary antigen, PCR). Briefly: Each serum sample was divided into 3 parts. To each part, the same volume of one of the 3 prepared solutions was added; for the first part – antigen Lp sg 1; for the second part – antigen Lp sg 3; for the third part – PBS. All samples were incubated for 18 hours at 4°C. After incubation, all samples were centrifuged and supernatants tested by ELISA IgM *L. pneumophila* sgs 1-7 and ImmuView™ test *L. pneumophila* sg 1 and 3.

Determination of sequence type (ST).

- **DNA extraction, PCR amplification.** Genomic DNA was extracted from 15 selected serum samples 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). Sequences were analysed using the online tool BLAST (http://blast.ncbi.nlm.nih.gov), or the 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 using 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 order: *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*.ST was represented by a number.

Statistical analysis. Multivariable analysis, correlations, sample comparisons, Anova tests were performed using Statgraphics Centurion v.XV.

RESULTS

Serological examinations. In total, positive results of IgM antibodies against *L. pneumophila* sgs 1-7 by ELISA (≥1.1) were found in 19/79 patients (24%), equivocal results (0.8–1.1) in 6 patients (7.6%). Positive results for *L. pneumophila* sg 1 (by immune-chromatographic, quick test) were found in 10 patients out of 63 examined (23/123 tested sera), positive results for *L. pneumophila* sg 3 were found in 10/63 patients and in 22/123 tested sera. However, positive results of ImmuView™ tests for sg 1 and for sg 3 were observed in different patients, only in 4 cases was the possibility of cross-reactions noted. Comparison of results obtained by ELISA IgM against *L. pneumophila* sgs 1-7 and immune-chromatographic quick test for *L. pneumophila* sg 1 and sg 3 (ImmuView™) indicated high correlation between the results (Po=0.0000), as well as in the analysis of tested serum samples in the examined patients (Tab. 2).

Table 2. Comparison of results obtained using two assays (ELISA and ImmuView™)

		Examined patients			Tested serum samples		
		ImmView sgs 1+3 Negative	ImmView sgs 1+3 Positive	In total	ImmView sgs 1+3 Negative	ImmView sgs 1+3 Positive	In total
ELISA IgM (sgs 1-7)	**Negative**	38	1	39	65	7	72
	Equivocal	4	2	6	8	5	13
	Positive	5	13	18	14	24	38
	In total	47	16	63	87	36	123

The percentage of *L. pneumophila* sero-positive patients differed and depended on the main recognition. The highest frequency of the positive level of IgM antibodies against *L. pneumophila* was among patients with pneumonia (ELISA sgs 1-7 – 35.5%, ImmuView sgs 1+3 – 30.8%) and exacerbation of asthma (26.6% and 36.4%, respectively) (Tab. 3).

Table 3. Serological examination results by test and group of patients

Patient group	No. of patients with antibodies IgM determined by different assays Positive/Equivocal/Negative (ELISA) or Positive/Negative (ImmuView™) (% Positive)			
	ELISA IgM *L. pneumophila* sgs 1-7	ImmuView™ *L. pneumophila* sg 1	ImmuView™ *L. pneumophila* sg 3	ImmuView™ *L. pneumophila* sg 1 and/or sg 3 (1+3)
Pneumonia	11/1/19 (35.5%)	6/20 (23.0%)	4/22 (15.4%)	8/18 (30.8%)
COPD	2/0/16 (11.1%)	1/11 (8.3%)	2/10 (16.7%)	2/10 (16.6%)
Exacerbation of asthma	4/1/10 (26.6%)	1/10 (9.0%)	3/8 (27.3%)	4/7 (36.4%)
URTI	2/4/9 (14.3%)	2/12 (14.3%)	1/13 (7.1%)	2/12 (14.3%)
In total	19/6/54 (24.0%)	10/53 (15.9%)	10/53 (15.9%)	16/47 (25.4%)

The sero-conversion was defined as a 4-fold increase/decrease of titre in paired sera tested by MAT or another test with serial dilution of serum samples. However, the definition of sero-conversion in paired sera tested by ELISA should be interpreted differently, because ELISA is based on measurement of absorbance – in a logarithmic scale. The dynamics of antibodies (increase or decrease of the IgM antibody level) were determined by the ratio of the highest and the lowest value determined by ELISA IgM *L. pneumophila* sgs 1-7 for one patient. In total, paired sera collected from 51 patients were examined by ELISA IgM *L. pneumophila* sgs 1-7. For the analysis, only pairs of sera (or 3 sera) with values ≥0.8 in at least one serum were selected. The IgM antibody ratio ranged from 1.1–42.9 in the pairs of sera. In 8 pairs of sera, the IgM antibody ratio was ≥ 2. Five of the 8 patients with paired sera were patients with pneumonia, 2 with URTI, and one person with COPD. However, there were no statistically significant differences between the determined ratio values in the tested pairs of sera, nor in the different groups of patients (Po>0.05).

Sero-conversion (change from negative to positive) was determined in 7 patients. In 5 out of 7 pairs of sera

collected from patients with sero-conversion, the ratio of IgM antibodies were ≥2. Dynamics of IgM antibodies in serum samples of the next 2 patients were also high (ratio values =1.5; 1.6) (Tab. 4).

Table 4. Dynamics of IgM antibodies against *L. pneumophila* sgs 1-7 (ELISA) and sero-conversion in 51 pairs of sera

Results in pair of sera	Seroconversion (by IgM ELISA *L. pneumophila* sgs 1-7)					
	No (both negative)	? (neg to equiv)	? (equiv to pos)	Yes (neg to pos)	? (both positive)	Total
No. of patients	27	2	8	7	7	51
Range of IgM ratio	-	1.9-2.0	1.3-2.2	1.5-42.9	1.1-2.0	1.1-42.9
No. of pairs with IgM ratio ≥2	-	1	1	5	1	8

Pre-absorption of serum samples by *L. pneumophila* sg 1 or sg 3 antigens. After pre-absorption (or dilution with PBS), sera were tested by the same serological tests. Results of the examination using ELISA IgM test for detecting antibodies specific to *L. pneumophila* sgs 1-7 were presented as index E1 (non-absorbed serum diluted with PBS/serum absorbed with antigen Lp sg 1), index E3 (non-absorbed serum diluted with PBS/serum absorbed with antigen Lp sg 3) and ratio E1/E3. The values of index E1 ranged from 1–20, and the values of index E3 ranged from 1-42. Index E1 was higher than index E3 in the examination of 5 serum samples – range from 1.25-11.2 times. Index E3 was higher than index E1 in 9 serum samples – range from 2.6-42 times (respectively, ratio E1/E3 from 0.4-0.02) (Tab. 5). In one serum sample, the values of indexes E1 and E3 were the same. In 8 absorbed serum samples, one of the indexes (E1 or E3) was higher than 10 (range from 12.6-42). The differences in the determined E1 and E3 were higher than 4-times in those sera, except for one (2.8 times, E1/E3=0.36). In five out of those 7 sera (collected from 4 patients) index E3 was higher than index E1 (5.3-42 times), and the probability of infection due to *L. pneumophila* sg 3 was suspected. In two patients, the probability of infection due to *L. pneumophila* sg 1 was based on ELISA results (E1/E3 = 11.0 and 11.2).

The results of the examinations using ImmuView™ test (sg 1/sg 3) indicated a possible *L. pneumophila* sg 1 infection in 5 patients (5 serum samples), and *L. pneumophila* sg 3 infection in 7 patients (8 sera). Analysis of the results of absorbed sera examinations showed a statistically significant correlation between both tests (ELISA and ImmuView™; Po = 0.0389), especially in sera with high values (≥10) of indexes E1 or E3 (Po=0.0111). Interpretation of the obtained results (determined serogroup) was the same in 7 out of 8 of the sera.

Genotyping of *L. pneumophila*. Amplifications (PCR) of 7 genes of *L. pneumophila* (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA)* were carried out directly in specimens collected from 13 patients (using DNA isolated from serum samples) without isolation of *L. pneumophila* strains. Obtained PCR products were sequenced and alleles determined. At least one amplicon was obtained in samples collected from 7 patients; however, only one product of amplification was found in 2 patients. All 7 amplicons were obtained (and sequenced) from samples collected from only one patient (Tab. 6). The sequence type (ST) was 36.

Alleles determined in serum samples of the next 6 patients varied. The profile of 5/7 alleles was the same in the sera of 2 patients: No. 69 (ST36) and No. 62 (only 5 alleles). Interpretations of absorption tests were unclear in both sera. Both patients were hospitalized because of pneumonia. Moreover, 6 out of 7 alleles were determined in a serum sample of one patient (No. 41) with upper respiratory tract infection. No similar profile was found among the rest of alleles.

Table 5. Results of serological examinations absorbed with *L. pneumophila* sg 1 or *L. pneumophila* sg 3 antigens serum samples from selected 13 patients.

Tested serum patient/ visit	Main symptoms	ELISA results before absorp	Results of ELISA after absorption			Interpr of ELISA results	Results of ImmuView sg 1 after absorption			Results of ImmuView sg 3 after absorption			Interprof ImV results
			Index E1 (NA/ Abs1)	Index E3 (NA/ Abs3)	Ratio E1/E3		ImVsg1 -NA (PBS)	ImVsg1 -Abs. Antigen Lp1	ImV sg3 -Abs. Antigen Lp3	ImVsg3 -NA (PBS)	ImV sg1 -Abs. Antigen Lp1	ImV sg3 -Abs. Antigen Lp3	
15/3	COPD	5.7	**1.84**	**6.5**	0.3	? (Sg 3?)	1	0.5	0	2	2	0	Sg 3
18/1	Asthma ex.	1.32	**1**	42	0.02	Sg 3	0	0	0	2	2	0	Sg 3
34/1	Pneumonia	1.8	**5.7**	**5.7**	1	?	0	0	0	1	1	0	Sg 3
41/2	URTI	1.5	**2.5**	**2**	1.25	?	1	0	1	1	0	0	Sg 1
47/2	Pneumonia	2.5	14.5	**1.3**	11.2	Sg 1	2	0	1	0	0	0	Sg 1
48/2	Pneumonia	1.4	**6.2**	**2.25**	2.75	?	1	0	1	0	0	0	Sg 1
53/2	Pneumonia	6	**2**	15.8	0.13	Sg 3	2	0.5	0	3	2	0	Sg 3
53/3		5.97	**2.4**	12.6	0.2	Sg 3	3	3	0	3	0.5	0	Sg 3
54/1	Asthma ex.	1.88	**1.8**	15.7	0.11	Sg 3	0	0	0	1	1	0	Sg 3
62/2	Pneumonia	1.33	**4.4**	**3**	1.5	?	1	0	1	0	0	0	Sg 1
63/1	Asthma ex.	2.86	**1.4**	23	0.06	Sg 3	0	0	0	1	1	0	Sg 3
69/2	Pneumonia	2.7	**3.4**	**9.4**	0.36	? (Sg 3?)	0	0	0	0	0	0	?
69/3		1.36	**5.6**	15.5	0.36	Sg 3	0	0	0	0	0	0	?
78/2	COPD	1.15	**3.1**	**8.1**	0.4	? (Sg 3?)	0	0	0	1	1	0	Sg 3
88/3	URTI	0.95	20	**1.8**	11	Sg 1	1	0	0.5	0	0	0	Sg 1

Table 6. Determined alleles (according to EWGLI SBT scheme) serogroups (by pre-absorption test) and other results.

Patient (No.)	Main recognition	Examinations conducted in serum samples						Respiratory sample	
		Profile of alleles	ST	Serogroup (based on pre-absorption)	ELISA IgM sgs1-7 (seroconversion/change of IgM level)	ImmView Lp1	ImmView Lp3	Chlamylege /PCR	Period&
34	Pneumonia	0;0;0;6,0;0;0		?	Pos (?/pos-equiv)	Neg	Weak	Pos	2
69	Pneumonia	3;4;1;1;14;9;1	36	?	Pos (?/pos-pos)	Neg	Neg	Pos	3
53	Pneumonia	2;0;0;1;0;4;0		Sg 3	Pos* (yes/neg-pos)	Pos*	Pos*	Pos	3
62	Pneumonia	3;4;0;1;14;0;1		?	Pos (yes/pos-neg)	Weak	neg	Pos	3
41	URTI	2;6;3;20;0;11;3		?	Pos (yes/neg-pos)	Weak	Weak	Pos	2
18	Asthma exacerbation	0;6;0;0;0;11;1		Sg 3	Pos (?/pos-equiv)	Neg	Pos	Nt	1
15	COPD	2;0;0;0,0;0;0		Sg 3	Pos* (yes/neg-pos)	Weak	Pos*	Nt	1

*Strong positive reaction
& period when respiratory samples were collected and tested with Chlamylege/PCR in the hospital (first – 1.01.2009 -8.06.2009; second – 8.06.2009-28.04.2010; third – 29.04.2010 – 31.10.2010).
Nt – not tested

DISCUSSION

According to the ECDC definition of legionellosis, this infection can be confirmed by at least one positive result in one of the following tests: isolation of *Legionella* spp. from clinical samples, detection of *L. pneumophila* antigens in urine or detection of specific antibodies against *L. pneumophila* sg 1 (seroconversion). The base for presumptive recognition of legionellosis is a positive result in one of the following tests; direct immunofluorescence assay, detection of *Legionella* genomes, antibody response to *L. pneumophila* non-sg1 or high single titre of antibodies specific to *L. pneumophila* sg 1 or *Legionella* spp. [1].

Diagnosis of *Legionella* infections, especially Legionnaire's disease, is mainly based on positive results of detection of antigens of *L. pneumophila* in urine (>80% per year) [1, 4, 5, 8, 9]. Serological tests are commonly used in the retrospective analysis because of difficulties in the interpretation of results and the need to test at least 2 samples collected within 2 – 6 weeks [10, 11, 12, 13]. However, IgM antibody detection has recently been shown as an early verification of *Legionella* infection [11]. Results of molecular methods (PCR, hybridisation) should be carefully considered and analysed in view of the possible presence of PCR inhibitors in clinical samples, or contamination of samples/reagents with *Legionella* spp. or their DNA [14]. Such a situation arose in a Warsaw hospital. The only method used for diagnosing *Legionella* infections was detection of *Legionella* spp. genomes in clinical samples by the Chlamylege test. According to procedure, in the case of positive results for *Legionella* spp., species specific hybridisation for *L. pneumophila* was performed. Unfortunately, examinations were very rarely carried out (1-2 per month), and no other method to confirm the results was used. After building renovation works at the hospital, the number of *Legionella* spp./*L. pneumophila* positive samples collected from respiratory tracts of patients increased significantly. Finally, almost all samples were positive. When the source of contamination was found, it turned out that the water used for pre-treatment of samples before adding Chlamylege reagents had been contaminated with *L. pneumophila* DNA, because the filters had not been changed after the renovation of the hospital. New filters and new reagents caused a rapid decrease of the number of *Legionella* spp. positive samples by molecular methods, although positive samples were still observed.

It should be noted that one of the side-effects was the increased number of examinations made based on suspicion of *Legionella* infection. However, not all *Legionella* positive Chlamylege results ought to be due to the effect of water contamination used for the preparation of respiratory sample or molecular examinations. The only available specimen applicable for excluding or confirming a *Legionella* infection was serum samples, previously examined for the detection of antibodies against other pathogens. Very high dynamics of IgM antibodies against *L. pneumophila* sgs 1-7 indicated infection caused by these bacteria in 8 patients. In 5 patients, an increase or decrease of the IgM antibody level from negative to positive was determined. The definition of seroconversion should be very carefully analysed and adapted to the used technique. The 4-fold increase/decrease of titre in paired sera tested with serial dilutions is considered as a significant result. However, one dilution of serum sample (usually 1:100) is tested with ELISA tests only. Moreover, the principles of reading in MAT and ELISA techniques are totally different. One publication has suggested a 2-time difference in ELISA tests to be significant [11].

In the presented study, 2 different serological tests were used for the detection of IgM antibodies against *L. pneumophila* sgs 1-7 (ELISA) and *L. pneumophila* sg 1 and sg 3 (ImmuView™, quick immunochromatographic test). The differences between results obtained by both tests might be caused by different specificities of the assays (detection of antibodies specific to a pool of 7 serogroups vs. 2 separate serogroups), as well as sensitivity because of the principle of the tests [11, 13]. However, the quick, simple and cheap test might be a response to the need for confirmation of PCR *Legionella* spp. positive results. Moreover, the probability of infection due to *L. pneumophila* sg 1 and/or sg 3 might be indicated using the ImmuView™ test. Pre-absorption of selected sera with antigens prepared from reference strains

L. pneumophila sg 1 and sg 3 confirmed the response to *L. pneumophila* sg 1 or to *L. pneumophila* sg 3 in 6 patients; however, the possibility of cross-reactivity of IgM antibodies against *L. pneumophila* serogroups in both tests should be considered. In any case, the main target, diagnosis of *Legionella* infection, was reached.

According to serological results, especially those based on the more detailed information from the ImmuView™ test, the possibility of 2 clusters were suggested: 4 patients infected at the same time with *L. pneumophila* sg 3, and 2 patients ill due to *L. pneumophila* sg 1. In order to confirm or exclude this thesis, the search for and genotyping of *L. pneumophila* was undertaken using DNA isolated directly from serum samples. Unfortunately, the serum samples had previously been defrosted a few times, creating a possibility of DNA degradation. In samples from 5 patients, the obtained amplicons were not good enough for sequencing. Determined profiles of alleles, both completed and not completed, indicated at least 4 sources of infections, but the possibility of one cluster is still being suggested. Detailed epidemiological examinations are required for determination of categories of infections (travel-associated, community acquired, or nosocomial). Moreover, these examinations might answer questions about the possibilities of outbreaks or sporadic cases. High prevalence of *Legionella* bacteria in the urban municipal water supply systems in the Lublin region (eastern Poland) described by Stojek et al. [15] indicated that risk of infection might be associated with exposure to water from many different sources. However, the possibility of using serum samples, especially when collected in the acute phase of disease, for detailed analysis, genetic analyses has also been presented in this study.

The presented study shows the usefulness of serological methods (with some modifications) in retrospective analyses of pseudo-outbreaks of legionellosis. Extended genetic analysis (SBT) performed directly in clinical specimens might be used for verification of other microbiological assays, and also for detecting an outbreak. In this study, the results of SBT suggested occurring sporadic *L. pneumophila* infections, non-hospital acquired. The presented study indicates the necessity to implement a complete scheme of diagnosing *Legionella* infections, consisting of different methods, in order to exclude any possibilities of false results (negative or positive), and to detect legionellosis in different stages of the disease [16]. Moreover, determination of cluster of legionellosis should be based on different methods of *Legionella* typing – serogrouping and genotyping (even not completed profile of alleles were obtained). Other authors also indicate the necessity for complex examinations of patients suspected of legionellosis, and high variation of serogroups/genotypes among isolated strains of *L. pneumophila*/*Legionella* spp. [17, 18, 19, 20, 21, 22, 23].

In our study, infections due to *L. pneumophila* were confirmed in patients with lower respiratory tract infections (pneumonia, COPD), as well as with asthma exacerbation or URTI. As shown by other research teams, infections caused by *Legionella* may be non-specific and without severe symptoms. The difference in the incidence rate of LD per 100,000 inhabitants in 35 countries (EWGLI members) indicates underestimation of all forms of legionellosis – LD (pneumonia) and non-pneumonia cases in Poland and some other countries. However, the difference in the incidence rates was connected rather with the different surveillance and notification systems than to average temperature or the number of sunny days. One of the crucial points causing the increased number of detected cases of legionellosis is the implementation of diagnostic tests in medical procedures. A study from Spain describes how the impact of the implementation of a urinary test for the detection of *L. pneumophila* sg 1 antigen increased the number of detected cases of legionellosis [8]. However, this study suggests that other serogroups may be an important clinical factor that is not diagnosed by urinary antigen test, and that perhaps LD is still underdiagnosed in countries where the urinary antigen tests are the only tests used for diagnosis of LD.

CONCLUSIONS

To summarize: the presented study indicates a strong underestimation of LD cases or extra-pulmonary forms of legionellosis in Poland; unawareness of the doctors being one of the most important reasons. None of the 8 patients with high dynamics of IgM antibodies specific to *L. pneumophila* was suspected of legionellosis by their doctors. Moreover, implementation of medical procedures for the differentiation of *Legionella* spp. from other aetiological agents causing atypical pneumonia is highly desirable in Poland.

It should be emphasized that the use of only one method for diagnosing *Legionella* infections may cause the occurrence of both false negative and false positive results. The possibility of applying a sequence based typing (SBT) directly on clinical specimens seems to be a promising method for diagnostics and epidemiological investigation.

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