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Development of a miniaturized DNA microarray for identification of 66 virulence genes of *Legionella pneumophila*

Opracowanie miniaturowej mikromacierzy DNA do identyfikacji 66 genów wirulencji *Legionella pneumophila*

Mariusz Żak[1ABDEF], Piotr Zaborowski[2AD], Milena Baczewska-Rej[3E], Aleksandra A. Zasada[3E], Renata Matuszewska[4E], Bożena Krogulska[4E]

[1] Department of Zoonoses and Tropical Diseases, Medical University of Warsaw, Żwirki i Wigury 61 Str., 02-091 Warsaw, Poland
[2] Department of Internal Diseases, Gastroenterology and Hepatology, University Hospital, University of Warmia and Mazury in Olsztyn, Oczapowskiego 2 Str., 10-719 Olsztyn, Poland
[3] Department of Bacteriology. National Institute of Public Health – National Institute of Hygiene. Chocimska 24 Str., 00-791 Warsaw, Poland
[4] Department of Communal Hygiene. National Institute of Public Health – National Institute of Hygiene, Chocimska 24 Str., 00-791 Warsaw, Poland

Summary

Introduction: For the last five years, *Legionella* sp. infections and legionnaire's disease in Poland have been receiving a lot of attention, because of the new regulations concerning microbiological quality of drinking water. This was the inspiration to search for and develop a new assay to identify many virulence genes of *Legionella pneumophila* to better understand their distribution in environmental and clinical strains. The method might be an invaluable help in infection risk assessment and in epidemiological investigations.

Material/Methods: The microarray is based on Array Tube technology. It contains 3 positive and 1 negative control. Target genes encode structural elements of T4SS, effector proteins and factors not related to T4SS. Probes were designed using OligoWiz software and data analyzed using IconoClust software. To isolate environmental and clinical strains, BAL samples and samples of hot water from different and independent hot water distribution systems of public utility buildings were collected.

Results. We have developed a miniaturized DNA microarray for identification of 66 virulence genes of *L. pneumophila*. The assay is specific to *L. pneumophila* sg 1 with sensitivity sufficient to perform the assay using DNA isolated from a single *L. pneumophila* colony. Seven environmental strains were analyzed. Two exhibited a hybridization pattern distinct from the reference strain.

Discussion: The method is time- and cost-effective. Initial studies have shown that genes encoding effector proteins may vary among environmental strains. Further studies might help to identify set of genes increasing the risk of clinical disease and to determine the pathogenic potential of environmental strains.

Key words: **DNA microarray • virulence factor • effector protein • *Legionella pneumophila* • legionellosis**

Streszczenie

Wprowadzenie: Przez ostatnie pięć lat coraz więcej uwagi w Polsce zaczęto poświęcać problemowi infekcji wywoływanych przez bakterie *Legionella* sp. Ma to związek m.in. z pojawieniem się nowych regulacji prawnych, dotyczących jakości wody przeznaczonej do spożycia przez ludzi. Było to inspiracją do opracowania testu umożliwiającego oznaczenie wielu genów wirulencji *Legionella pneumophila* w celu lepszego zrozumienia ich dystrybucji w szczepach środowiskowych i klinicznych. Metoda może być nieocenioną pomocą w skuteczniejszym przewidywaniu ryzyka wywołania choroby przez dany szczep i stanowić nowe narzędzie podczas dochodzeń epidemiologicznych.

Materiał/Metody: Mikromacierz bazuje na systemie Array Tubes, zawiera trzy kontrole pozytywne i jedną negatywną. Geny docelowe kodują elementy strukturalne T4SS, białka efektorowe oraz geny wirulencji niezwiązane z systemem sekrecji. Sondy zaprojektowano używając programu OlogoWiz, mikromacierz analizowano z użyciem programu IconoClust. W celu izolacji szczepów klinicznych i środowiskowych zgromadzono próbki BAL i pobrano próbki wody z różnych, niezależnych instalacji ciepłej wody w budynkach użyteczności publicznej.

Wyniki: Zaprojektowano i wyprodukowano mikromacierz do identyfikacji 66 genów *L. pneumophila* biorących udział w patogenezie. Czułość mikromacierzy pozwala na analizę DNA wyizolowanego z pojedynczej kolonii *L. pneumophila*. Analizowano 7 szczepów środowiskowych, z których dwa różniły się wzorem hybrydyzacji od szczepu referencyjnego.

Dyskusja: Prezentowana metoda nie jest czasochłonna i kosztowna. Analiza szczepów środowiskowych wykazała możliwe różnice w obecności genów kodujących białka efektorowe. Dalsze analizy mogą pozwolić na identyfikację genów zwiększających ryzyko wywołania choroby oraz na ocenę zjadliwości szczepów.

Słowa kluczowe: **mikromacierz DNA • czynnik wirulencji • białko efektorowe • *Legionella pneumophila* • legioneloza**

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Author's address: mgr Mariusz Żak, ul. Niepodległości 17/22, 05-600 Grójec; e-mail: mariuszzakster@gmail.com

Abbreviations: **AFLP** – amplified fragment length polymorphism; **BAL** – broncho-alveolar lavage fluid; **BLAST** – basic local alignment search tool; **EWGLI** – European working group for *Legionella* infections; **LCV** – *Legionella* containing vacuole; **MLVA** – multiple-locus variable number of tandem repeats analysis; **NSI** – normalized signal intensity; **PCR** – polymerase chain reaction; **PFGE** – pulsed-field gel electrophoresis; **SBT** – sequence-based typing; **T4SS** – type IV secretion system.

Introduction

Legionella spp. are causative agents of legionnaire's disease. For most infections *Legionella pneumophila* sg 1 is responsible [55]. Water is the major reservoir for *Legionella*e. *Legionella*e have been detected in natural waters (e.g. ground water, surface water, hot spring water) and manmade aquatic environments (e.g. hot and cold water system, cooling towers and evaporative condensers, respiratory therapy equipment). Conditions in manmade aquatic environments that are favorable for the amplification of *Legionella* growth include temperatures of approximately 25-42oC, stagnation, scale and sediments, biofilms and the presence of protozoa. *Legionella*e survive in aquatic environments as intracellular parasites of free-living protozoa [20]. The main transmission route of *Legionella* is inhalation of droplets of water- air aerosol containing bacteria. Occasionally, inhaled by humans it multiplies in alveolar macrophages [26]. After uptake to the host cell via phagocytosis, *L. pneumophila* starts to export effector proteins through T4SS [9]. More than 200 potential effectors have been identified using bioinformatic methods [8]. Many various assays have been developed to confirm the translocation of putative effectors. The methods apply cya-fusion assay, immunofluorescence microscopy, inter-bacterial transfer, SidC-based translocation assay, and fusion to β-lactamase. The methods have also been used to identify effectors not selected previously by bioinformatics. Discovered proteins affect vesicle trafficking, regulate host GTPases, inhibit apoptosis, mimic host ubiquitination pathways and induce a stress response [5,10,12,14,19,29,30,33,39,40,49].

After infection the disease may develop up to 20 days. This means that identification of the source of infection is difficult, especially if the disease developed after travel. To establish the link between environmental and clinical strains, genetic studies are required. Routinely PFGE, considered as a gold standard, SBT, AFLP and MLVA are used. These techniques take advantage of identification of DNA polymorphism after restriction enzymes digestion (PFGE, AFLP), analysis of multiple locus variable number tandem repeats (MLVA) and sequencing of selected genes (SBT) [21,22,41,51,60]. The methods have different ability to discriminate strains based on the presence in the genome of DNA polymorphisms unspecific for virulence, but do not allow particular virulence genes to be identified. The technique which allow this are microarrays. The method has been used for the rapid identification of antimicrobial resistance genes in Gram-negative bacteria [6], genotyping of enterohemorrhagic *Escherichia coli* (EHEC) [24] and *Staphylococcus ureus* [32], detection of herpesvirus and adenovirus co-infections [38] and for haemagglutinin subtyping and pathotyping of avian influenza viruses [23]. Simultaneous identification of many virulence genes is essential, because effector proteins of *L. pneumophila* display a high level of redundancy. Deletion of a single gene often does not cause a noticeable effect on phenotype [17]. Identification of many virulence genes in environmental strains will give a more reliable view on the virulence potential of a given strain and will help to better understand their distribution. In future it may help to predict with higher accuracy risk of infection.

Risk assessment of infection is usually made on the basis of the number of *Legionella* sp. in tested water. Routinely, for quantitative analysis, membrane filtration and cultivation on solid microbiological media is performed. The method in detail is described in PN – EN ISO 11731-2: 2008 [45]. In Polish law a regulation that relates directly to this issue is the decree of Ordinance of the Minister of Health of March 29, 2007 on the requirements related to the quality of water intended for human consumption [47,48]. European guidelines, formed by EWGLI in 2005, contain many instructions about risk assessment and management in order to minimize risk of infection [27]. Attachment No. 7 to the Polish decree defines the risk based on the number of *Legionella* sp. determined according to PN – EN ISO 11731-2: 2008. The risk gradation is consistent with the EWGLI guidelines, but the regulation says nothing about risk assessment and management performed individually for each installation. The assessment of risk of infection based on the number of *Legionella* sp. is very difficult, because infection dose is still not determined and not all individuals exposed will develop the disease.

To date, five genomes of *L. pneumophila* have been sequenced. Pan-genome analysis reveals that only 67% of identified genes are common for all strains, while 33% are strain-specific [15]. Such high genetic diversity might be very useful for pathotyping of a strain.

This was the inspiration for our study, in which we present a low-density oligonucleotide DNA microarray dedicated to identification of 66 virulence genes of *Legionella pneumophila* sg 1. Genes of interest are involved in translocation of effector proteins into the eukaryotic cell (dot/icm genes encode structural elements of T4SS, and proteins with function similar to molecular chaperones), growth on solid microbiological media, intracellular growth, inhibition of apoptosis, maturation of LCV, iron acquisition, and cytochrome c synthesis. Function of many target genes encoding effector proteins is not determined. Target genes encoding effector proteins were selected on the basis of studies where their translocation to a eukaryotic cell was proven. Target genes are listed in Table 1. All probes and primers were designed in this study.

Materials and Methods

Microarray design and manufacture

The designed microarray is based on the Array Tube system (Alere Technologies GmbH). On the surface 9 mm2 of glass slide probes are immobilized via amino-linker C7. Each slide has its own hybridization chamber. In the first step of the detection procedure, products of linear PCR, labeled with biotin, hybridize to specific probes. During the next step, streptavidin conjugated with horseradish peroxidase binds to the biotin. The enzyme transforms the soluble and colorless substrate into an insoluble black product. In the last step each microarray is analyzed in an array reader and clarity of all spots is calculated.

All probes were designed using OligoWiz 2.0 software. *Legionella pneumophila* subsp. pneumophila str. Philadelphia 1 genome was used as a reference sequence. Probes were designed in two stages. In the first stage, a set of probes specific to each target gene was designed (up to 15 probes for each gene). Probe sequences were selected to be specific for the target gene of the reference strain and to have specified GC contents, lengths, and Tm in order to yield high hybridization efficiency. In the second stage the BLAST algorithm was used to select one probe specific to the consensus region of the target gene.

Primers were designed using Primer-BLAST. Primers were selected to be specific and to have similar GC contents, lengths, and Tm in order to have similar template binding efficiency. Probes and primers are specific to a consensus region of target genes. The primer binding site is situated up to 100 bp upstream of the probe-binding site (Figure 1). Probes and primers are listed in Table 2.

Each target gene has one specific probe and each spot is duplicated. Spot distance: 180 μm, spot diameter: 150 μm (Figure 2). The microarray has one negative control (NC) – a probe and primer complementary to human papilloma virus E7 gene – and 3 positive controls – probes and primers complementary to the gene encoding 16S rRNA (lpg0302), *mip* gene (lpg0791) encoding macrophage infectivity potentiator, and *pgi* gene (lpg0759) encoding glucose-6-phosphate isomerase.

Bacterial strains

Legionella pneumophila was isolated from water samples collected from the hot water systems according to PN – EN ISO 11731-2: 2008. Serogroup 1 was identified using an *L. pneumophila* latex kit (BIORAD) according to the manufacturer's instructions. (Five strains were obtained from the Department of Communal Hygiene, National Institute of Public Health – National Institute of Hygiene, Warsaw. Two

Table1. Genes of interest

Effector proteins			
No.	**Protein**	**Gen ID**	**Reference**
1	LegA11	lpg0436	[18]
2	LegA12	lpg0436	[18]
3	LegA15	lpg2456	[18]
4	LegA2	lpg2215	[18]
5	LegA3	lpg2300	[18]
6	LegA8	lpg0695	[18, 44]
7	LegAS4	lpg1718	[18, 25]
8	LegAU13	lpg2144	[18]
9	LegC3	lpg1701	[18, 42]
10	LegC4	lpg1953	[18]
11	LegC5	lpg1488	[18]
12	LegC6	lpg1588	[18]
13	LegC7	lpg2298	[18]
14	LegC8	lpg2862	[18]
15	LegG1	lpg1976	[18]
16	LegG2	lpg0276	[18]
17	LegL1	lpg0945	[18]
18	LegL2	lpg1602	[18]
19	LegL3	lpg1660	[18]
20	LegL7	pg2400	[18, 44]
21	LegLC4	lpg1948	[18, 25]
23	LegS2	lpg2176	[18, 42]
24	LegU1	lpg0171	[18, 19]
25	LegU2	lpg2830	[18]
26	LepA	lpg2793	[18]
27	LepB	lpg2490	[18, 19]
28	LidA	lpg0939	[18]
29	SdhA	lpg0376	[18]
30	SidG	lpg1355	[18, 19]
31	SidH	lpg2829	[18]
32	SidJ	lpg2155	[18]
33	VipA	lpg0390	[18, 28]
34	VipD	lpg2831	[49]
35	VipF	lpg0103	[49]
36	VpdA	lpg2410	[53]
37	VpdB	lpg1227	[29]
38	WipA	lpg2718	[43]
39	WipB	lpg0642	[33]
Structural elements of T4SS			
No.	**Protein**	**Gen ID**	**Reference**
40	DotB	lpg2676	[57]
41	DotC	lpg2675	[54]
42	DotD	lpg2674	[54]
43	IcmB(DotO)	lpg0456	[1]
44	IcmC(DotE)	lpg0453	[54]
45	IcmD(DotP)	lpg0454	[13]
46	IcmE(DotG)	lpg0451	[54]
47	IcmF	lpg0458	[52]
48	IcmG(DotF)	lpg0452	[54]
49	IcmH(DotU)	lpg0459	[52]
50	IcmJ(DotN)	lpg0455	[54]
51	IcmK(DotH)	lpg0450	[54]
52	IcmL(DotI)	lpg0449	[1]
53	IcmM(DotJ)	lpg0448	[1]
54	IcmO(DotL)	lpg0446	[31]
55	IcmP(DotM)	lpg0445	[54]
56	IcmQ	lpg0444	[50]
57	IcmR	lpg0443	[50]
58	IcmT	lpg0441	[35]
59	IcmW	lpg2688	[9]
60	IcmN(DotK)	lpg0447	[58]
Other virulence factors			
No.	**Protein**	**Gen ID**	**Reference**
61	CcmC	lpg0858	[56]
62	IraA	lpg0747	[55]
63	IraB	lpg0746	[55]
64	LetA	lpg0747	[16]
65	Mip	lpg0791	[3]
66	RpoS	lpg1284	[36]

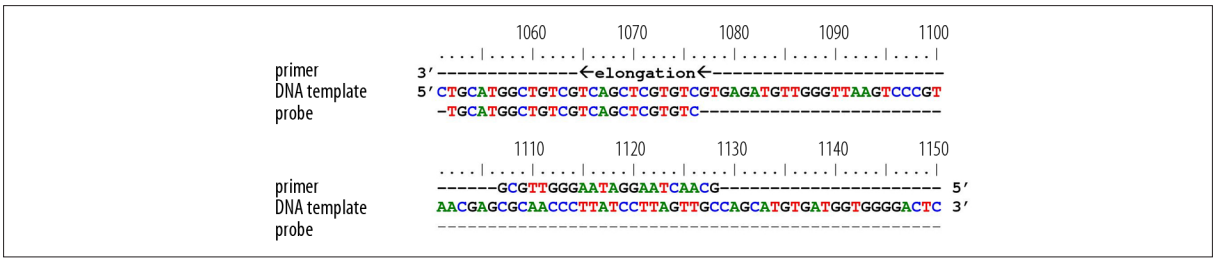

Fig. 1. Principle of the method. Location of hybridization sites of probes and primers on a DNA template. Arrow indicates the direction of elongation.

Table 2. Names and sequences of all designed probes immobilized on the microarray and primers used in linear multiplex PCR

Probe name	Sequence 5′-3′	Primer name	Sequence 5′-3′
ccmC	TTGTATGCCTGGATGGGGTTTCTGGC	ccmC-p	AGCCCTGCCATTTTGATACG
dotB	TTAGCGGGGAAGAGCGTTTGGGAAGA	dotB-p	GCCAAATGCAAAGCCTAATTGT
dotC	GCGAGATGGCCCTTAAGGAAACCGC	dotC-p	GCCTGTTTGTTTAATTGCTCGTC
dotD	TGCAAGCAAGAGCCAGTGTCGATTGGT	dotD-p	CTGAAATGTGCTGCTTTGGC
icmB(dotO)	GGCATCTGGGACCAAGTGAAGCTCGA	icmB(dotO)-p	GTACCATTTGGGAACAGTGCA
icmC(dotE)	GCTGATCCGACTTGTACTGGCGGCAC	icmC(dotE)-p	TTTGAACAGGCTCCAACTGATT
icmD(dotP)	ACAGCGGGCTCTTACTTGGCTGGATT	icmD(dotP)-p	GGGTTATCCTTGTGCTGCTTAAA
icmE(dotG)	GGAAAGGCATGGAGTCAACAGGCGC	icmE(dotG)-p	AAAATACCCAAACCTGTACCAGAA
icmF	TTGAAGTGAATAGCAACTCCGGCCGCT	icmF-p	CCCATTTAGAATTCCTGGAGTAAAAG
icmG(dotF)	AATTCCAGGTCGGGCTTGGCTAATTGG	icmG(dotF)-p	CCTGGAATTTTTGATCCCTCTC
icmH(dotU)	ACCATCTGAAAGCAGACGGGCGACAAT	icmH(dotU)-p	GCGATGTGGTTTATTAAAACGG
icmJ(dotN)	TCAATTTTGCGGGTTTCAAGCCCGATT	icmJ(dotN)-p	AATCACCATCCAGATTCACGA
icmK(dotH)	ATATTGTCGCCGGGCTGGTTAGCCAG	icmK(dotH)-p	TAAGCATGCGTTCCATCTGC
icmL(dotI)	GGCTTTTTTACTGCGGAAGGCTGGGAC	icmL(dotI)-p	TTTTCGCTTTAACCGCATCA
icmM(dotJ)	ATGCGACCAGCGGCATAACTCCTCC	icmM(dotJ)-p	CAATGCTTCAGATGACATGTTAGGA
icmO(dotL)	GTAGTGCCTGCGCAAGCTCGTTCTC	icmO(dotL)-p	TGTCCAGCAAAAATAGCAGAAAA
icmP(dotM)	GCGTGCGGTCGGTGATTATATGCGTT	icmP(dotM)-p	AGAACAAAGGCTAACACTACCAG
icmQ	AACGGATAGGTGCCGCTAGTCAGGC	icmQ-p	TCGATTTGCAAGATGAGACTCTG
icmR	TGATGACAGTGCACGAAATCCATTTGGT	icmR-p	CCTATCTCTTTAACACGAGGTGG
icmT	CCACTGGCGTGACTCAGCGAGAAG	icmT-p	AGAAATAAAAATATTGGGAATGCGG
icmW	GCCTGATTTAAGCCATGAAGCCTCCGC	icmW-p	TGATGACCCTGTAAATCATGGG
pgi	TGAATTGCTGGCTGGTGCCCATGA	pgi-p	ACCGATTAAAGCCATTAGCACAG
legA2	GGCTATTTCACGCGGCTTTGGAGAGC	legA2-p	CCTTTCATGGAAAACAAGGCAT
legA3	TGATTCTTCCTGCGGCGAGTCGTG	legA3-p	ACTGGTGATAACGGACAAAGC
legA8	TTCGAGATGCCAATGGCCGTACCTTT	legA8-p	GCGGTAAACCTTGTTGCATTAAA
lepA	TGAGCCGGAATCTTCAGAGGCTTTGG	lepA-p	CCATATGCAGCATGCCTAAAAA
lepB	TAAAGGCCGCACAGATGATCCCGC	lepB-p	TGATGCCATTCACGGATTTTAG
lidA	AGTGCGACCCTACCCTGCCAATCTGA	lidA-p	CAGTCAATTTCAGCTGGGAACA
dotK	CGTGTTGATGGTGCATGCGATGCA	dotK-p	TTTGGCCTACTGATGCAACTTT
mip	GGCATGCAAGACGCTATGAGTGGCG	mip-p	AACGTCTTTCATTTGCTGTTCG
rpoS	CGCGTTCGCCAAATTCAAGTTGAAGC	rpoS-p	CCAACCCGCTCTAACAAAGTT
sdhA	TGAACACCAGGAATGCACCAAGCGTT	sdhA-p	AATGCCTGTAACTTCTCCTGC
sidG	AACGCAAGTTACGCGGAAAGCCCAA	sidG-p	GTTCATAAGCCTTCTCGTGGGT
sidH	ATCGCTACCGGGGCATCAGGGATT	sidH-p	TTGCGGATTGATAGTCTTCAGC
sidJ	GCACTCATGACACCTCTCTGCCACCA	sidJ-p	AGCGCCCACTTAACACCTT
vipA	AATTATGCGCGCAATGGCACCCC	vipA-p	TCATGGTTTTGTTCCTTTGCC

Table 2 continued. Names and sequences of all designed probes immobilized on the microarray and primers used in linear multiplex PCR

Probe name	Sequence 5′-3′	Primer name	Sequence 5′-3′
vipD	CCTGTTCAAAGGCCCTGCCCAAGA	vipD-p	TTCCTGATTTTCAGAGGTAGAAACC
vipF	TTTGAGCGAAGGCAAATCCCGGGT	vipF-p	TTTTGAATATGAAATCCAAGCTGAA
iraA	AAATCCTCGATCTGGGCGTTGGCAA	iraA-p	CATCAATTCCTGTGAAATTGGCCA
letA	TGCCTGGGATAGATGGGTGGGAAGTG	letA-p	GGAGAGGATCTGAGCAAATAGCT
legC8	TTGCAAACGCATGCCTGTGTGGCT	legC8-p	GGATTGTGTACGCTCAACTTC
vpdA	CAAATACTGAGGCGACATGCCCATCA	vpdA	GATTGGTCAAAGCAATAGTTGGC
vpdB	AGCGATGGAGCATGTCATGAGGATGG	vpdB-p	CAGAGATTCAGGAAAATGCGTG
wipA	TCACTGGGCGACTTGCATGGCAAT	wipA-p	TGACGGAAAAGAAAATGGATAAGC
wipB	CAGGCAGATTACTGGGGGACCGAAGC	wipB-p	GATTTCTTCCAAACGCTCCTGT
legA11	GCCCCCATTTCAGAACAAGCACCAC	legA11-p	TGATATTGGTGCTATTCACCAGC
legA12	TAATATGCCGTGGCGCTGAACCACC	legA12-p	GACCGCTTTTAAGAAAGCAACC
legAS4	AATCCTGGCGATGGTTGGCTATCGG	legAS4-p	AGGGCATTTGTTCTAATCGGTA
legAU13	TGGGGAAATCTTATGGTGGCGGCA	legAU13-p	TTGACCTCTTCCAGGTTATTGC
legC3	GGGTTAAACGCGCTCAGGCAAGAATTG	legC3-p	GAATACTGCGCAACAAAATGGA
legC4	TGCAAATATCGCAGGGAGCGAGCAG	legC4-p	CATGTCGCGCATTGAGTTTT
legC5	GCGGTTTCAGAAGCTCACCAGGATGCT	legC5-p	TAGCAAAATACTCAGCGCCAAA
legC6	TGGCTCTCAATGGTGCTCAGGCTGG	legC6-p	GAACTGCCTGGAGGTATTTTG
legC7	TGGACGACCACCATACATGCAATGTGA	legC7-p	CCCTTCTTTTAATCGTTTGGCA
legG1	ATGGCCAATTGGGATGCGGGGATA	legG1-p	AGTGGCGACCATTTCTACTGA
legG2	CGGATGAAGATCCGAAACCAGTGCTTG	legG2-p	GCTCACGCATCAATTTCATACC
legL2	GGCAGTTCTGCTCAGTATGCTCAAGGC	legL2-p	ATTCAGTTCCAGTCTTTTGCCC
legL3	AATGCAACAAAGAATGGGGCATTCGCT	legL3-p	GATAGAGGGCCTGATAAATCCAAA
legL7	GGCAGCCATCCCTTTCACCATCACC	legL7-p	TTCTGAGCTGCTTTTACTGCTGA
legLC4	AGGACAGGCATCGAATTGGCCGC	legLC4-p	AACTCCAGAATTTTTGAGGGCA
legLC8	TGGTGATGAGGGTGTATCCAGGCTTGC	legLC8-p	CCCCCTATTCCACAATTATCCA
legS2	CATTCGCGCAAGGGCAAAAGGCAT	legS2-p	CTCCAGTTAATTCGGCTGCTTT
legU1	GCAAAATACGACCCCACAGAGCCTGG	legU1-p	GAGCTAGTTTTGATTTGGCATCAAG
legU2	CGGGTTGCTGCACGCCAAAAAGAATA	legU2-p	TGGGCAGTAATGACAGGTGTT
legL1	ATATTGGTCCGGAGGGTGCTCAATGGC	legL1-p	CCTCGTCACCAATCTCGTTAAA
iraB	TGTGATGCACGTGCGATTGCCTTTG	iraB-p	GACCCAACCTGCAATTCGTC
legA15	TGGTTATGACAGCGCACAAAGCGCA	legA15-p	CCTTCTGAGTTTGTGGCTTGAA
16S rRNA	TGCATGGCTGTCGTCAGCTCGTGTC	16S rRNA-p	GCAACTAAGGATAAGGGTTGCG
NC	TTCAAAGGGGAACGTCCCGCCAGT	NC-p	GAAGGCAGCTCCGAAGAAAA

strains were isolated in this study.) *Legionella pneumophila* sg 1 Philadelphia 1 (ATCC 31152) was used as a reference strain. In order to isolate clinical strains of *L. pneumophila*, BAL samples were collected from 31 patients with chronic cough, hemoptysis, chest pain, diarrhea, respiratory disorders, abdominal pain, confusion. Samples were cultivated on BCYE-α with cysteine and confirmed on BCYE-α without cysteine and BMPA plates. Not a single strain was isolated. To confirm the negative result of the cultivation, DNA from BAL samples was isolated and used as a template for real time PCR reaction to detect and quantify *Legionella* sp. (BioRad). In all tested samples *Legionella* sp. DNA was not detected.

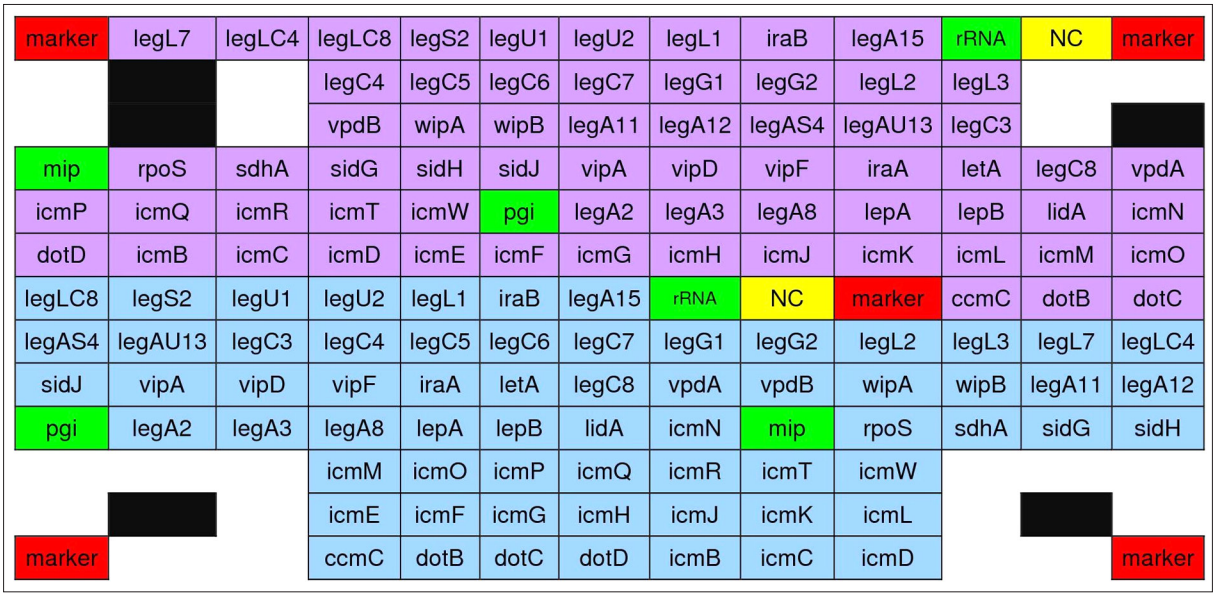

Fig. 2. Layout of oligonucleotide probes on the microarray. Each spot is duplicated. Violet and blue color correspond to two areas of the microarray. In each area one duplication of each spot is placed. Rectangles coded marker (red color) correspond to spots with immobilized biotin (positive controls for Poly-HRP-streptavidin). Each microarray has 5 marker spots. Black rectangles correspond to reference points of the microarrays. Green rectangles corresponds to positive control spots. Yellow rectangles corresponds to negative control spots.

DNA isolation and preparation

One colony of each strain of *L. pneumophila* was resuspended in lysis buffer from DNeasy Blood & Tissue Kit and the DNA was isolated according to the manufacturer's instructions. BAL samples (5 ml) were centrifuged (10 min., 12000 rpm), the pellet was resuspended in lysis buffer from DNeasy Blood & Tissue Kit and the DNA was isolated according to the manufacturer's instructions. DNA concentration was measured using a spectrophotometer. DNA isolated from bacterial strains was subsequently diluted in double-distilled water to the expected concentration. DNA isolated from BAL samples was concentrated to the expected concentration using BlueMATRIX PCR/DNA Clean-up Purification Kit (Eurex) according to the manufacturer's instructions.

Labeling and PCR

Each elongation reaction contained 1 µl dNTP mix (Fermentas), 1 µl Terminator DNA polymerase buffer 10× (New England Biolabs), 0.1 µl Terminator DNA polymerase (New England Biolabs), 0.35 µl Biotin-16-dUTP (Roche), 1 µl primer mix (Genomed), and 1 µg genomic DNA (up to 6.55 µl). The PCR conditions were as follows: 3 min at 96°C, followed by 40 cycles of 20 s at 51°C, 40 s at 72°C and 60 s at 96°C.

Hybridization and detection

Each Array Tube was in the first step washed with 500 µl of double-distilled water and incubated at 55°C using a thermomixing device for 5 min at 550 rpm. In the next step the water was discarded and 500 µl of hybridization buffer was added for 5 min at 55°C and 550 rpm. The hybridization buffer was removed from the hybridization chamber and 100 µl of a hybridization mix (90 µl of hybridization buffer and 10 µl of PCR product) was added for 60 min at 55°C and 550 rpm. In the next step the Array Tube was washed once with 500 µl of 2 × SSC with Triton X-100 (10 × SSC is 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 5 min at 30°C and 550 rpm, once with 500 µl of 2 × SSC for 5 min at 30°C at 550 rpm and finally with 500 µl of 0.2 SSC for 5 min at 20°C and 550 rpm. The washing buffer was discarded and 100 µl of blocking solution was added (blocking solution is 6 × SSPE containing 0.005% Triton X-100 and 2% milk powder; 6 × SSPE is 60 mM sodium phosphate, 1.08 M NaCl, 6 ml EDTA, pH 7.4) for 15 min at 30°C and 550 rpm. Blocking solution was removed and horseradish peroxidase-streptavidin conjugate solution was added (Poly-HRP-streptavidin was diluted 1: 3000 in 6 × SSPE with Triton X-100) for 15 min at 30°C and 550 rpm. To remove excess HRP, the Array Tubes were washed using washing buffers as described above. In the last step, 100 µl of peroxidase substrate was added (Seranum Grün, Seranum diagnostica GmbH) and the staining reaction was performed at ambient temperature without shaking for 10 min. Array Tubes were placed in an Array Tube reader (Alere Technologies GmbH). Data were acquired and analyzed using IconoClust 3.2 software (Alere Technologies GmbH).

Data analysis

Analysis was performed using IconoClust 3.2 software. Raw data were normalized and the Normalized Spot Intensity (NSI) was calculated. NSI=1-(M/BG) where M represents the average intensity of a spot and the BG represents the intensity of the local background. NSI value >0.1 of a given spot was considered to be "positive".

Results

Assay validation

In the first step of the validation, quality control of the designed and manufactured microarrays was performed. To exclude contamination of probes with biotin, the full

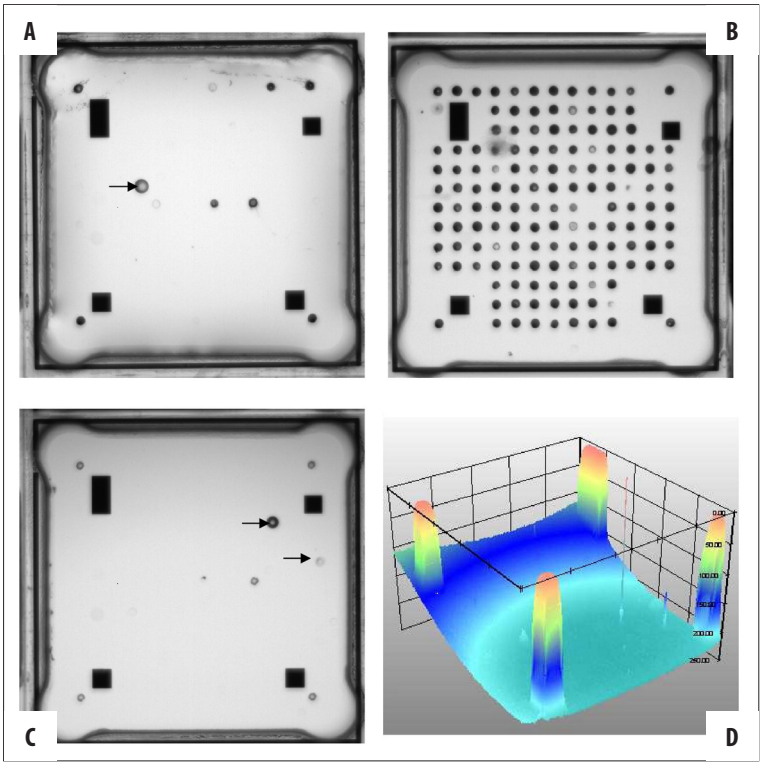

Fig. 3. Images of the microarray after hybridization procedure. Each arrow points to an air-bladder. (**A**) *E. coli* DNA used as a PCR template. (**B**) *L. pneumophila* DNA used as a PCR template. (**C**) DNA isolated from BAL sample used as a PCR template. (**D**) 3D visualization of background signal of the microarray before data normalization. Distilled water was used instead of PCR product and the full hybridization procedure was performed.

hybridization procedure was performed using double-distilled water in place of biotinylated PCR product. NSI of spots corresponding to the *legL1* gene was higher in comparison with background and other spots on the microarray. In further analysis these spots were not considered because of possible contamination with biotin. All data were normalized to minimize the effect of heterogeneous background signal emission (Figure 3D).

Specificity of the microarray was tested using genomic DNA isolated from *Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1, *Escherichia coli* (O157: H7), *Pseudomonas aeruginosa*, *Haemophilus influenzae* and DNA isolated from BAL samples. The PCR product used for hybridization, where DNA of *Escherichia coli* (O157: H7), Pseudomonas aeruginosa and *Haemophilus influenzae* was the template, hybridized only to the probes corresponding to 16S rRNA (Figure 3A). The PCR product from BAL samples gave no positive signal in any spot area (Figure 3C). The PCR product from the *L. pneumophila* reference strain hybridized to all probes corresponding to target genes. The spot corresponding to the icmL probe characterized low value of the NSI in all tested microarrays. The shape of the spot indicates that probes were incorrectly spotted during the manufacturing process. Hybridization to probes corresponding to negative control was not detected (Figure 3B).

Assay sensitivity

To determine the minimal DNA concentration for the analysis, a series of dilutions of reference DNA was made: 250 ng/µl, 150 ng/µl, 100 ng/µl and 50 ng/µl. The detection limit was 975 ng DNA/reaction. It is the equivalent of 2.66×10^{8} DNA copies, based on a genome size of 3.4×10^{6} nucleotide residues corresponding to a 3.6×10^{-15} g/1 genome copy. In experiments with DNA dilutions near to the detection limit, heterogeneous signals from different spots were observed. To overcome this effect, 1.2 µg of DNA should be used in routine experiments.

Tested strains

In the last stage of the study we tested DNA isolated from 7 environmental strains. Analysis of DNA isolated from five strains showed a positive signal in all spots corresponding to target genes. DNA from two tested strains, named LPE06 and LPE07, gave a spot signal pattern different to the reference strain and the other environmental strains (Figure 4). Exact analysis of spot signal level indicated that both strains give a positive signal of spots corresponding to dot/icm genes. Strain LPE06 gives no signal of spots corresponding to *legC8*, *legLC4*, *legU2*, *sidG*, *SidH* and *wipB* genes. Strain LPE07 gives no signal of spots corresponding to *legA12*, *legC4*, *legC8*, *legL7*, *legLC4*, *legU1*, *legU2*, *lepB*, *sidG*, *sidH*, *vipD* and *wipB* genes.

Discussion

Routinely, legionellosis risk assessment is made on the basis of the number of *Legionella* sp. detected in a water sample using microbiological methods. Results of the analysis determine actions which should be put into practice to reduce the level of contamination. These actions are often expensive and cause difficulties in usage of the installation. If dead ends are present, effective chemical or thermal disinfection is very difficult to perform. The method also has limitations. Identification of *L. pneumophila* in water from cooling towers has shown that the number of bacteria may vary in a short period of time. Single water testing may not give reliable information about the level of contamination of installation [7]. Other studies have

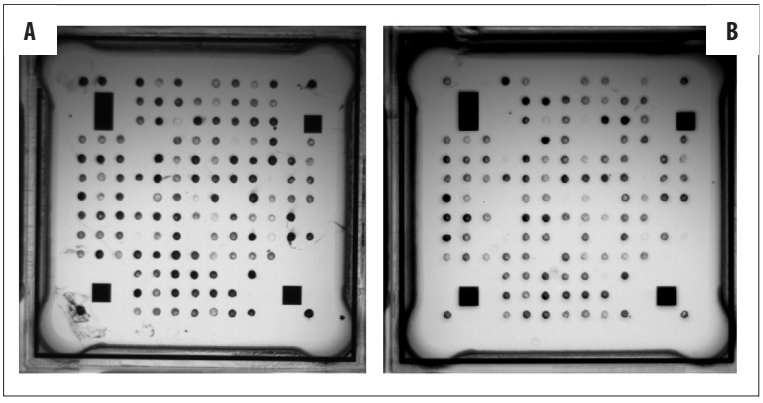

Fig. 4. Images of the microarray after hybridization procedure. (**A**) DNA of LPE06 strain was used as a PCR template. (**B**) DNA of LPE07 strain was used as a PCR template.

shown that genotypes of *L. pneumophila* in cooling towers do not change while the number of bacteria vary [46]. For this reason, the bacteria number count should not be the only method for the assessment of risk of infection. The microbiological method should be supplemented by analysis of virulence potential of a strain, which is determined by specific virulence genes.

The presented microarray allowed identification of two strains with a hybridization pattern different to the reference strain. Strain LPE07 does not give a positive signal of spots corresponding to genes encoding 6 effector proteins and strain LPE07 to 12 effector proteins. Previous studies with microarrays have shown that genes *sidG* and *sidH* are not highly conserved. Gene *sidG* was present in 52% of tested strains while *sidH* was present in 72%. Genes encoding structural elements of T4SS were present in all tested strains except the gene icmX, which was present in 65% of tested strains [11]. These data are compatible with the results of our study, because all spots corresponding to dot/icm genes give a positive signal. Strains LPE06 and LPE07 give no positive signal for three and seven eukaryotic-like genes respectively. Leg genes have been discovered recently and their function in many cases is still not known. Further analysis of clinical and environmental strains isolated from different installations made of different materials with parallel identification of protozoa will help to better understand their distribution and functions in pathogenesis. Highly conserved virulence genes are important for survival of *Legionella*. Strains with deletions in these genes may have reduced ability to infect protozoa and therefore to survive in water with biocides and to infect humans. Knowledge about the virulence potential of a strain identified in an installation of interest with information about the number of bacteria will help to choose the most effective intervention.

Sensitivity of the array allows one to analyze DNA isolated only from one colony and is similar to sensitivity levels reached in other studies with this technology [2,4,37]. Furthermore, there are several advantages of this technology. In one experiment detection of up to 100 genes in duplicate is possible in only 8 hours. It is less costly and time-consuming than 200 PCR reactions. The Array Tube system allows one to perform the assay with standard laboratory equipment, because each Array Tube has the Eppendorf tube format. Moreover, PCR is prone to contamination, which is almost impossible with the Array Tube system, and a free PCR product environment is not rigorously required. It is an open system and changes in microarray layout are easy to introduce. In future the method might be a supplementation of the microbiological method in routine diagnostics of environmental samples.

Studies performed at the National Institute of Public Health – National Institute of Hygiene showed that the bacteria were present in most tested samples collected from hot water distribution systems in inpatient healthcare facilities [34]. Moreover, no method of disinfection applied once guarantees permanent elimination of the pathogen. Only constant monitoring will give valid information about the risk of infection. The microbiological method has limitations and because of that the microarray was designed. The technique has been used in many fields of environmental and medical diagnostics and it is also a promising method for identification and graduation of risk of infection of *Legionella pneumophila*.

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