Human Anti-Lipopolysaccharid (LPS) antibodies against *Legionella* with high species specificity

Philipp Kuhn^{a,g}, Stefanie Thiem^{b,c}, Michael Steinert^{b,d}, Duncan Purvis^e, Veronika Lugmayr^f, Ulrich Treutlein^f, Lutz Plobner^f, Robert-Matthias Leiser^f, Michael Hust^a and Stefan Dübel^{a,*}

^a*Biotechnology and Bioinformatics, Institute for Biochemistry, Technische Universität Braunschweig, Braunschweig, Germany*

^b*Institute for Microbiology, Technische Universität Braunschweig, Braunschweig, Germany*

^c*Department of Animal Breeding and Genetics, University of Veterinary Medicine Vienna, Vienna, Austria*

^d*Helmholtz Center for Infection Research, Braunschweig, Germany*

^e*Vantix[®] DIAGNOSTICS, Lowell, MA, USA*

^f*Agrobiogen GmbH, Hilgertshausen, Germany*

^g*YUMAB GmbH, Rebenring, Braunschweig, Germany*

Abstract. *Legionella* are Gram-negative bacteria that are ubiquitously present in natural and man-made water reservoirs. When humans inhale aerosolized water contaminated with *Legionella*, alveolar macrophages can be infected, which may lead to a life-threatening pneumonia called Legionnaires' disease. Due to the universal distribution of *Legionella* in water and their potential threat to human health, the *Legionella* concentration in water for human use must be strictly monitored, which is difficult since the standard detection still relies on lengthy cultivation and analysis of bacterial morphology. In this study, an antibody against *L. pneumophila* has been generated from the naïve human HAL antibody libraries by phage-display for the first time. The panning was performed on whole bacterial cells in order to select antibodies that bind specifically to the cell surface of untreated *Legionella*. The bacterial cell wall component lipopolysaccharide (LPS) was identified as the target structure. Specific binding to the important pathogenic *L. pneumophila* strains Corby, Philadelphia-1 and Knoxville was observed, while no binding was detected to seven members of the families *Enterobacteriaceae*, *Pseudomonadaceae* or *Clostridiaceae*. Production of this antibody in the recombinant scFv-Fc format using either a murine or a human Fc part allowed the set-up of a sandwich-ELISA for detection of *Legionella* cells. The scFv-Fc construct proved to be very stable, even when stored for several weeks at elevated temperatures. A sensitivity limit of 4,000 cells was achieved. The scFv-Fc antibody pair was integrated on a biosensor, demonstrating the specific and fast detection of *L. pneumophila* on a portable device. With this system, 10,000 *Legionella* cells were detected within 35 min. Combined with a water filtration/concentration system, this antibody may be developed into a promising reagent for rapid on-site *Legionella* monitoring.

Keywords: Phage display, panning, antibody engineering, antibody fragments, *Legionella*, LPS, biosensor, diagnostics

1. Introduction

The first outbreak of the Legionnaires' disease was recorded in 1976. During a convention of the American Legion at the Bellevue Stratford Hotel in Philadelphia, 182 people contracted the disease. 147 of the victims had to be hospitalized and 29 died. The patients

*Corresponding author: Stefan Dübel, Technische Universität Braunschweig, Institute for Biochemistry, Biotechnology and Bioinformatics, Spielmannstr. 7, 38106 Braunschweig, Germany. E-mail: s.duebel@tu-bs.de.

suffered from a severe pneumonia, accompanied by fever, chills, coughs, headaches or diarrhea [15]. The causative agent was named after the clinical symptoms of the first victims – *Legionella pneumophila* [7]. *Legionella* are flagellated Gram-negative bacteria that belong to the class of gamma-proteobacteria. They are ubiquitously present in water habitats, including natural and man-made reservoirs [14,37] where they infect and parasitize protozoa [1,45]. In the host, a specialized compartment is formed, that enables replication of *Legionella* [6].

Humans are infected with *Legionella* by inhalation of aerosols [13]. Thus, outbreaks are linked to aerosol producing devices like air conditioners [20], showers [46], spa pools [11] or cooling towers [2]. Once having entered the human lung, alveolar macrophages are infected by *Legionella*, using the same infection strategies as in protozoa [17]. This can lead to a local inflammation and onset of the Legionnaires' disease. At least 63 species or subspecies of the genus *Legionella* were identified so far. Among them, the serogroup 1 of *L. pneumophila* is the most prevalent pathogen with 84% of all clinical cases [49].

Since 2001, the Robert Koch-Institute has been registering an increasing number of *Legionella* infections in Germany. In 2013, approximately 1,000 cases were reported with a lethality rate of 5% [44]. But it is expected that at least 15,000 to 30,000 infections occur each year that are not recognized by clinicians due to the unspecific symptoms and lack of routine testing in hospitals [47].

Due to the ubiquitous distribution of *Legionella* in man-made water systems and their potential threat to human health, the German government decided in 2001 to monitor the *Legionella* levels in drinking water (TrinkwV 2001). If the *Legionella* concentration exceeds 1 cfu/mL it is regarded hazardous, demanding disinfection and reporting to health authorities.

Up to now, the standard method for the detection of *Legionella* in water is microbiological cultivation followed by analysis of microbial morphology. However, this method is time-consuming [5], requires trained laboratory staff and harbors the risk of under-/over estimation of *Legionella* if the sample is not treated properly [33]. Therefore, there is a need for a fast, sensitive, and easy manageable diagnostic point-of-care test. These requirements can be met by antibody-based immunosensors as demonstrated for other pathogens [32,35].

In this work, recombinant monoclonal antibodies were selected from the naive human phage display li-

braries HAL9/10 [30] by panning on whole *L. pneumophila* cells. Sandwich pairs were designed and used in portable immunosensor for the detection of *Legionella*.

2. Materials and methods

2.1. Preparation of cells for antibody selection

After washing of a *P. aeruginosa* (NCTC 10662) culture with PBS, cells were inactivated for 2.5 h at room temperature by formaldehyde treatment (0.1% v/v). The *L. pneumophila* (str. Corby) [24] culture was washed with PBS and directly used for panning.

2.2. Antibody selection

Phage ($\sim 1 \times 10^{11}$ cfu) of the HAL9/10 libraries [30] were incubated with the formaldehyde fixed *P. aeruginosa* cells ($\sim 1 \times 10^9$ cells) for 1 h at room temperature in suspension. Cell bound phage were removed by centrifugation (10,000 g, 5 min), the supernatant was collected and cleared from the remaining cells by filtration (0.2 μ m). Living *Legionella* cells ($\sim 1 \times 10^9$ cells) were diluted in BSA solution (2%) and added to the supernatant containing the preabsorbed phage library. After incubation for 1 h at room temperature, weakly bound or unbound phage were removed by washing with PBS-T (0.05%). *Legionella* bound phage were rescued by infection of *E. coli*, amplified and used for another panning round [16]. In total, three panning rounds were performed. At the end of the third round, *E. coli* cells were infected with selected phage and plated to select single clones as described before [16].

2.3. Screening-ELISA

The cavities of a 96-well microtiter plate were filled with 2YT-GA (150 μ L/well) and inoculated with single colonies of the third panning round. After propagation of cells, scFv expression was induced by adjusting to 50 μ M IPTG for 16 h at 30°C. After clearance by centrifugation, the scFv containing supernatant was screened for cell binding by ELISA.

For this purpose, *L. pneumophila* (str. Corby) and *P. aeruginosa* cells were diluted in sodium carbonate buffer (pH 9.7) and immobilized for 16 h at 4°C to the cavities of ELISA-plates (4×10^7 cells/well). After blocking and washing with PBS, scFv supernatants were added. Bound scFv were detected with the Myc1-9E10 antibody in combination with a secondary HRP-conjugated antibody, and TMB as substrate measured at 450 nm.

Fig. 1. Identification of monoclonal human scFv antibodies to *L. pneumophila* after panning. Whole cell ELISA on immobilized *L. pneumophila* and textit*P. aeruginosa* cells of clones enriched by panning of HAL9/10. Shown are the results of 80 clones picked after the first panning campaign. Similar results were obtained for the other pannings. Neg. ctr.: cultivation medium.

2.4. Production of scFv-Fc

The DNA encoding the scFv was subcloned in two eukaryotic scFv-Fc expression vectors (pCSE2.6), containing either a human or murine Fc part, as described before [23]. For production of scFv-Fc, HEK293-6E cells were transiently transfected. Supernatant was harvested after 7 days and antibodies were purified by Protein A affinity chromatography. The protein concentration was determined by UV/VIS spectrometry and purity was determined by SDS-PAGE and Coomassie staining of samples. Binding of the antibodies was tested to non-, heat-, formaldehyde- and freeze/thaw treated *L. pneumophila*, *P. aeruginosa* and *E. coli* cells by ELISA as described above, but using human-Fc or mouse-Fc specific secondary antibodies for detection.

2.5. Target identification

Whole cell lysate was prepared by incubation of *Legionella* ($\sim 1 \times 10^9$ cells) for 20 min at 95°C in Laemmli buffer (200 μ L). For preparation of a protein reduced lysate, *Legionella* ($\sim 1 \times 10^9$ cells) were first incubated for 15 min at 95°C in Urea (4 M). Then, Proteinase K (20 μ g) was added and incubated for 2 h at 37°C. The phenol-water extract was prepared as described before [12]. The cell preparations were separated by SDS-PAGE and either Coomassie stained or blotted on a PVDF membrane. After blocking of the membrane, 20 μ g scFv-Fc (PHK121-H2h) was added. Bound scFv-Fc were detected with a secondary AP-conjugated antisera that catalyzed NBT/BCIP substrate reaction.

2.6. Detection of *L. pneumophila* by sandwich-ELISA

The murine scFv-Fc variant was immobilized to the cavities of ELISA-plates (400 ng/well) for 1 h at room temperature. After blocking of wells with BSA (1%) and washing with PBS-T (0.05%), various concentrations of heat-treated *L. pneumophila* (str. Corby) and

E. coli cells were added. After washing with PBS-T (0.05%), the captured cells were detected with the human scFv-Fc variant (400 ng/well) as described above. For specificity assays, whole cell lysates of *Legionella* and various Gram-negative bacterial strains were prepared or cells were heat-treated for 95°C for 30 min and compared by sandwich-ELISA and immunoblot as described above.

2.7. Stability measurement of PHK121-H2 for long term storage

The PHK121-H2h antibody was diluted to the final assay concentration in BSA (1%). Then, aliquots were prepared and stored for different time intervals at 4°C, RT or 37°C. Activity of the antibodies was measured by ELISA binding to whole *L. pneumophila* cells (str. Corby) as described before.

2.8. Immunosensor measurement

Electrochemical VR2 sensors (Vantix) were used according to the providers recommendations. In brief, the murine scFv-Fc variant (150 ng) was immobilized on the sensors for 15 min at 37°C in a humidity chamber. After washing, sensors were coated with Stabilizing-Film, dried for 2 h at 37°C and stored afterwards at 4°C. Then, sensors were activated in BSA (1%) before a dilution series of heat-treated *L. pneumophila* (str. Corby) or *E. coli* cells were added on the sensor. The captured cells were detected with the human scFv-Fc variant (0.5 μ g/ml) in combination with a secondary HRP-conjugated antibody. The sensors were then transferred into Reading-Solution and the HRP activity on the sensors was recorded online.

3. Results

3.1. Human antibody generation by phage display

To isolate *Legionella* specific antibodies, three in-

Fig. 2. Binding of PHK121-H2h to different cell preparations. Whole cell ELISA on *L. pneumophila*, *P. aeruginosa* and *E. coli* that were either (a) non-, (b) heat-, (c) formaldehyde- or (d) freeze/thaw treated. Binding of the scFv-Fc with human Fc part (PHK121-H2h) is shown.

dependent pannings of the human naïve scFv libraries HAL9/10 were performed on whole cells in suspension. To remove sticky and cross-reactive binders, the antibody phage libraries were first preincubated on BSA and formaldehyde-fixed *P. aeruginosa*. Non-bound antibody-phage were then selected on living *L. pneumophila*. After three panning rounds, around 600 clones were selected, used for the production of soluble monoclonal scFv antibody fragments and screened for specific cell binding on *Legionella* by ELISA (Fig. 1). In total, seven scFv clones were identified to bind specifically to *Legionella* but not to *Pseudomonas* cells with a signal to noise ratio of better than three. DNA sequence analysis of the isolated clones revealed that scFv PHK121-H2, was always selected in the independent pannings. This scFv clone also showed the strongest signal in ELISA and was chosen for further engineering. The monovalent scFv fragment PHK121-H2 was converted to a bivalent scFv-Fc fusion protein, carrying either a human (PHK121-H2h) or murine (PHK121-H2m) Fc part to facilitate its use in a sandwich-ELISA. Production of the scFv-Fc variants of PHK121-H2 in HEK293-6E cells, followed by Protein A affinity chromatography, resulted in yields

of 105 and 122 mg per L of culture volume and purities of 98% and 95% for the human Fc and mouse Fc versions, respectively.

Dilution series of PHK121-H2h and PHK121-H2m were tested in ELISA on whole cells of immobilized *L. pneumophila*, *P. aeruginosa* or *E. coli* cells that were either non-, heat-, formaldehyde- or freeze/thaw treated. Specific binding of the antibody was seen to all *Legionella* cell preparations but not to the other bacteria (Fig. 2). This was the first indication that the target on the cell surface may not be a protein. No significant differences were observed for the antibody versions carrying either human or murine Fc parts.

3.2. Identification of the *Legionella* antigen bound by PHK121-H2h

Since the panning was done on whole cells, the antigen recognized by PHK121-H2h was unknown. PHK121-H2h staining of western blots of total cell lysate of *L. pneumophila* showed multiple bands in the range of 25 to 65 kDa that formed a ladder-like pattern described to be typical for LPS [25]. To confirm that the antigen is not a protein, the experiment

Fig. 3. Identification of the antigen band by PHK121-H2h. SDS-PAGE analysis of whole cell lysate, Proteinase K-treated cell lysate and an aqueous-phenol extract of *L. pneumophila* by (a) Coomassie staining and (b) immunoblot staining with PHK121-H2h.

was repeated on Proteinase K-treated cell lysate. No difference in staining was observed, indicating binding to a non-protein target like LPS (Fig. 3). To further exclude residual proteins, a hot-phenol/water extraction of *L. pneumophila* cells was performed as described for the purification of LPS [12]. No proteins were detected in this preparation, yet reactivity with PHK121-H2h was still unchanged. The hypothesis that PHK121-H2h binds to LPS is also consistent with the resistance to chemical and physical denaturation conditions observed in ELISA before.

3.3. Detection of *L. pneumophila* by sandwich-ELISA

Direct ELISA is sensitive to impurities, which may hamper the analysis of diverse environmental specimens. Therefore, we developed a sandwich-ELISA with the aim to improve both specificity and sensitivity by specific capturing of cells. Here, *L. pneumophila* was first captured from a 2% BSA suspension with immobilized PHK121-H2m, carrying the murine Fc part. After that, the human Fc version PHK121-H2h was used for detection of captured bacteria. Detection of *L. pneumophila* could be demonstrated down to a biomass that represents approximately 4,000 cells ($N = 4$, $P < 0.0001$, calculated by unpaired t test with Welch's correction) (Fig. 4).

3.4. Specificity of PHK121-H2h

So far, PHK121-H2h binding was only analyzed for the Corby strain of *L. pneumophila*. In order to evaluate the antibody's specificity in more detail, binding to a panel of six other *Legionella* strains and seven other Gram-negative bacteria that are commonly found in

Fig. 4. Detection of *L. pneumophila* by sandwich-ELISA. Dilution series of heat treated *L. pneumophila* and *E. coli* cells were analyzed in a sandwich-ELISA, using the murine Fc version of PHK121-H2 for capture and its human Fc version for detection. Detection of *L. pneumophila* but not *E. coli* was observed. The black arrow indicates the detection limit that was determined to a biomass corresponding to 4000 cells ($N = 4$, $P < 0.0001$).

water, was tested by immunoblot staining on cell lysate and by sandwich-ELISA on heat treated cells. Both methods revealed binding to the *L. pneumophila* strains Corby, Philadelphia-1 and Knoxville, which were all isolated from infected humans and belong to serogroup 1 (Fig. 5). Weak binding was also detected by ELISA but not by immunoblot to an environmental isolate of *L. pneumophila* that belonged to serogroup 1. No binding was observed to another environmental strain of serogroup 6 and to the species *L. micdadei*. Additionally, no binding to *Citrobacter freundii*, *Escherichia coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Raoultella planticola*, *Clostridium bifermentans* and *Clostridium perfringens* was detected (Fig. 5).

Despite LPS was considered to be the antibody target, high specificity of the antibody was measured in particular for *L. pneumophila* from serogroup 1 which

Fig. 5. Specificity of PHK121-H2h. Specificity of PHK121-H2h by (a) sandwich-ELISA and (b) immunoblot staining on different strains of *L. pneumophila* and various Gram-negative bacteria. ELISA was done as in Fig. 1; immunoblot was done as in Fig. 2.

were isolated from infected humans and thus expected to be very pathogenic.

3.5. Detection of *Legionella* using an immunosensor

To demonstrate that a fast and sensitive *Legionella* detection with PHK121-H2h is technically feasible on a portable device, an immunosensor was developed based on the electrochemical VR2 system from Vantix. First, stability of PHK121-H2h for long-term storage was assessed, a parameter important for the conceived use in point-of-care applications. For this purpose, the antibody was diluted to final assay concentration in 2% BSA and stored for up to 10 weeks at 4°C, RT or 37°C before binding to *Legionella* was measured by ELISA. Storage for 10 weeks at 4°C resulted in no reduction of the binding signal. 93% and 88% reactivity was re-

Fig. 6. Stability of PHK121-H2h. After dilution of the antibody to the final assay concentration, aliquots were stored for different time intervals at 4°C, RT or 37°C. Binding to *L. pneumophila* was measured by ELISA as described in Fig. 2.

tained after storage for 10 weeks at RT and 37°C, respectively (Fig. 6).

Fig. 7. Detection of *L. pneumophila* on the Vantix immunosensor system. Dilution series of heat treated *L. pneumophila* and *E. coli* were captured with PHK121-H2m and detected with PHK121-H2h using the Vantix VR2 system. Specific detection of *L. pneumophila* but no binding to *E. coli* was detected.

The Vantix biosensor assay was set up similar to the sandwich-ELISA. PHK121-H2m was immobilized and used for cell capture, while PHK121-H2h was used in combination with a secondary HRP conjugated antibody for detection. The catalytic activity of the enzyme created a shift in the electrochemical potential on the sensor surface that was recorded in real time. Analysis of the electric potential after 60 seconds revealed that a biomass corresponding to 10,000 *Legionella* cells could be easily distinguished from the background generated by *E. coli* (Fig. 7). The detection limit is comparable to the ELISA results, but was obtained within 35 min in a portable system.

4. Discussion

Since 2001, *Legionella* testing of drinking water is mandatory in Germany and regulated by the *Trinkwasserverordnung* (TrinkwV 2001). If more than 1 cfu/ml water is detected, local health authorities have to be informed and disinfection methods applied. Up to now, *Legionella* detection is done by the cultivation method which is regarded as gold standard. Yet, this requires several days to be conclusive. Faster alternatives were developed, including quantitative PCR [29], fluorescence *in situ* hybridization [8], flow cytometry [3] or ELISA [43]. In this work, we generated a human monoclonal recombinant antibody against *L. pneumophila* by antibody-phage display and applied it for specific detection of *Legionella* in a portable immunosensor.

So far, several reports demonstrated the successful use of antibody-phage display for the generation of diagnostic antibodies for the detection of pathogenic

bacteria [31]. But to our knowledge, this is the first report of a successful selection of a recombinant human antibody against the pathogen *L. pneumophila* from a naïve human antibody library by antibody-phage display. The use of whole cells for panning resulted in a highly specific antibody which recognized a non-proteinaceous antigen which can be assumed to be cell wall lipopolysaccharides (LPS). LPS is of particular interest as a detection target, as it's abundance promises good sensitivity [38]. Second, it is highly stable [34], allowing detection even after a harsh sample treatment, which is helpful since water samples usually have to be concentrated significantly before detection. Third, it is the serogroup determining antigen of *Legionella* [10,39] that contains conserved as well as variable epitopes. That allows to do sero- and subtyping of *Legionella* as demonstrated previously [21]. The O-chain of *Legionella* LPS consists of a homopolymer of legionaminic acid [26] that exhibits distinct acetylation patterns. These are expected to mediate serologic specificity and antibody binding [28]. Since environmental samples of *L. pneumophila* serogroup 1 and 6 as well as *L. micdadei* were not detected sensitively, it might be possible that the antibody binds to a virulence marker of serogroup 1, as described previously with Mab3/1 [22]. In order to prove this hypothesis, a broader testing on other *Legionella* strains would be necessary. Significantly, no cross-reactivity was observed to seven other bacteria usually found in water samples, including members of *Enterobacteriaceae*, *Pseudomonadaceae* and *Clostridiaceae*. Using the sandwich-ELISA, a detection limit of 4,000 *Legionella* cells was reached, which would require a significant volume reduction of water samples to be of practical use. The necessary sample concentration units are under development, for example by using monolithic affinity filtration [41] or tangential flow filtration [9].

The novel human antibody to *Legionella* compares favorably to LPS specific *Legionella* antibodies generated by Hybridoma technology. For example, clone II-6-18 that is specific for *L. pneumophila* serogroup 1 showed a detection limit of 30,000 cells in ELISA [19, 42]. Clone 53-3D12 binds to a common epitope on LPS, allowing detection of *L. pneumophila* serogroup 1 to 8 [4]. Unfortunately, no reports were found where this antibody was used for quantitative detection of *Legionella* in a sandwich set-up, which might indicate that the antibody was not suited for this application. For example, stability and cross-reactivity issues are a problem often reported for Hybridoma de-

rived antibodies (Bradbury et al., in preparation). Compared to that, our scFv-Fc antibody exhibited high stability, even when stored in finally diluted form at 37°C. A very similar approach to our work using antibody phage-display was done by Griep et al. who used purified LPS of *Ralstonia solanacearum* Race 3 for antibody selection [18]. The selected antibodies were used in combination with a polyclonal capture antiserum in order to detect bacterial cells by sandwich-ELISA. High sensitivity of 5×10^3 cfu/ml was reached, but a polyclonal antiserum was still needed, adding the disadvantages of limited resource and complex undefined composition. Furthermore, this LPS antibody cross-reacted with other organisms, rendering very much its relevance for diagnostics [18].

Another antibody-based sensor system was recently described by Wunderlich [48], who used monolithic adsorption filtration (MAF) with centrifugal ultrafiltration (CeUF) to concentrate 10 L of water to 1 mL. The concentrated sample was applied by microfluidics on a microarray (CL-SMIA), where the *Legionella* cells were captured by polyclonal or monoclonal LPS antibodies. The detection was performed with a biotinylated polyclonal antibody in combination with a HRP conjugate. Based on the number of bound cells, a chemiluminescent signal was generated and optically analyzed. Using this system, a detection limit of approximately 1,500 cells was reached. Regarding that these cells were present in 10 L water, a sensitivity of 0.39 cfu/mL was achieved [48]. This is the first indication that a sensitivity of 1 cfu/mL can be reached which is required for *Legionella* diagnostic by law. Combinations of improved water concentration and biosensors may provide a practical solution in the future. Also, the affinity of recombinant antibodies can be quite reliably improved by in vitro evolution [27]. Furthermore, our antibody shows specificity only to *Legionella pneumophila* of serogroup 1. This group contains with 84% of all infections the most important pathogenic *Legionella* [49]. Yet, since all *Legionella* species are regarded as potential pathogenic [40], a diagnostic test requires broad specificity to different *Legionella* serogroups and species. Thus, the specificity of a point of care test device needs to be extended to other subtypes, serogroups or species. As demonstrated recently, a single amino acid exchange in the CDR region can be sufficient to alter the antibody's specificity to *Legionella* serotypes [36]. This may allow specificity expansion of PHK121-H2 by random mutagenesis and re-selection by phage display.

To our knowledge, this is the first report that demonstrated successful generation of a potentiometric im-

munosensor for the detection of *L. pneumophila*. Using this portable technology, the assay time could be reduced to 35 min with a detection level still comparable to the tedious sandwich-ELISA.

Acknowledgments

This project was funded by Eurostars Project E! 6994. We would like to thank Doris Meier and Saskia Helmsing for excellent technical support.

References

- [1] Y.A. Kwaik, L.Y. Gao, B.J. Stone, C. Venkataraman and O.S. Harb, Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis, *Appl Environ Microbiol* **64** (1998), 3127–3133.
- [2] D.G. Addiss, J.P. Davis, M. LaVenture, P.J. Wand, M.A. Hutchinson and R.M. McKinney, Community-acquired Legionnaires' disease associated with a cooling tower: Evidence for longer-distance transport of *Legionella pneumophila*, *Am J Epidemiol* **130** (1989), 557–568.
- [3] H. Aurell, P. Catala, P. Farge, F. Wallet, M. Le Brun, J.H. Helbig, S. Jarraud and P. Lebaron, Rapid detection and enumeration of *Legionella pneumophila* in hot water systems by solid-phase cytometry, *Appl Environ Microbiol* **70** (2004), 1651–1657.
- [4] C. Barthe, J.R. Joly, D. Ramsay, M. Boissinot and N. Benhamou, Common epitope on the lipopolysaccharide of *Legionella pneumophila* recognized by a monoclonal antibody, *J Clin Microbiol* **26** (1988), 1016–1023.
- [5] A. Borges, M. Simões, A. Martínez-Murcia and M. Saavedra, Detection of *Legionella* SPP in natural and man-made water systems using standard guidelines, *Journal of Microbiology Research* **2** (2012), 95–102.
- [6] J.A. Bozue and W. Johnson, Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: Uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion, *Infect Immun* **64** (1996), 668–673.
- [7] D.J. Brenner, A.G. Steigerwalt and J.E. McDade, Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, familia nova, *Ann Intern Med* **90** (1979), 656–658.
- [8] S. Buchbinder, K. Trebesius and J. Heesemann, Evaluation of detection of *Legionella* SPP in water samples by fluorescence in situ hybridization, PCR amplification and bacterial culture, *Int J Med Microbiol* **292** (2002), 241–245.
- [9] L. Cai, Y. Yang, N. Jiao and R. Zhang, Evaluation of tangential flow filtration for the concentration and separation of bacteria and viruses in contrasting marine environments, *PLoS One* **10** (2015), e0136741.
- [10] C.A. Ciesielski, M.J. Blaser and W.L. Wang, Serogroup specificity of *Legionella pneumophila* is related to lipopolysaccharide characteristics, *Infect Immun* **51** (1986), 397–404.
- [11] N. Coetzee, H. Duggal, J. Hawker, S. Ibbotson, T.G. Harrison, N. Phin, V. Laza-Stanca, R. Johnston, Z. Iqbal, Y. Rehman, E. Knapper, S. Robinson and N. Aigbogun, An outbreak of Legionnaires' disease associated with a display spa pool in retail premises, Stoke-on-Trent, United Kingdom, July 2012, *Euro Surveill* **17** (2012).

- [12] M.R. Davis, Jr. and J.B. Goldberg, Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction, *J Vis Exp* (2012).
- [13] R.B. Fitzgeorge, A. Baskerville, M. Broster, P. Hambleton and P.J. Dennis, Aerosol infection of animals with strains of *Legionella pneumophila* of different virulence: Comparison with intraperitoneal and intranasal routes of infection, *J Hyg (Lond)* **90** (1983), 81–89.
- [14] C.B. Fliermans, W.B. Cherry, L.H. Orrison, S.J. Smith, D.L. Tison and D.H. Pope, Ecological distribution of *Legionella pneumophila*, *Appl Environ Microbiol* **41** (1981), 9–16.
- [15] D.W. Fraser, T.R. Tsai, W. Orenstein, W.E. Parkin, H.J. Beecham, R.G. Sharrar, J. Harris, G.F. Mallison, S.M. Martin, J.E. McDade, C.C. Shepard and P.S. Brachman, Legionnaires' disease: Description of an epidemic of pneumonia, *N Engl J Med* **297** (1977), 1189–1197.
- [16] A. Frenzel, J. Kügler, S. Wilke, T. Schirrmann and M. Hust, Construction of human antibody gene libraries and selection of antibodies by phage display, *Methods Mol Biol* **1060** (2014), 215–243.
- [17] L.Y. Gao, O.S. Harb and Y.A. Kwaik, Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa, *Infect Immun* **65** (1997), 4738–4746.
- [18] R.A. Griep, C. van Twisk, J.R. van Beckhoven, J.M. van der Wolf and A. Schots, Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum* race 3, *Phytopathology* **88** (1998), 795–803.
- [19] J.G. Guillet, J. Hoebeke, C. Tram, S. Marullo and A.D. Strosberg, Characterization, serological specificity, and diagnostic possibilities of monoclonal antibodies against *Legionella pneumophila*, *J Clin Microbiol* **18** (1983), 793–797.
- [20] D. Harper, Minimising the spread of Legionnaires' disease – a programme designed to prevent incubation of LP in hospital water and air conditioning systems, *Hosp Eng* **38** (1984), 5–9.
- [21] J.H. Helbig, J.B. Kurtz, M.C. Pastoris, C. Pelaz and P.C. Luck, Antigenic lipopolysaccharide components of *Legionella pneumophila* recognized by monoclonal antibodies: Possibilities and limitations for division of the species into serogroups, *J Clin Microbiol* **35** (1997), 2841–2845.
- [22] J.H. Helbig, P.C. Luck, Y.A. Knirel, W. Witzleb and U. Zahringer, Molecular characterization of a virulence-associated epitope on the lipopolysaccharide of *Legionella pneumophila* serogroup 1, *Epidemiol Infect* **115** (1995), 71–78.
- [23] V. Jager, K. Bussow, A. Wagner, S. Weber, M. Hust, A. Frenzel and T. Schirrmann, High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells, *BMC Biotechnol* **13** (2013), 52.
- [24] R.I. Jepras, R.B. Fitzgeorge and A. Baskerville, A comparison of virulence of two strains of *Legionella pneumophila* based on experimental aerosol infection of guinea-pigs, *J Hyg (Lond)* **95** (1985), 29–38.
- [25] D. Jurgens and F.J. Fehrenbach, Cross-reacting lipopolysaccharide antigens in *Legionella pneumophila* serogroups 1 to 14, *Infect Immun* **63** (1995), 2180–2184.
- [26] Y.A. Knirel, E.T. Rietschel, R. Marre and U. Zahringer, The structure of the O-specific chain of *Legionella pneumophila* serogroup 1 lipopolysaccharide, *Eur J Biochem* **221** (1994), 239–245.
- [27] N. Kobayashi, H. Oyama, Y. Kato, J. Goto, E. Soderlind and C.A. Borrebaeck, Two-step in vitro antibody affinity maturation enables estradiol-17 β assays with more than 10-fold higher sensitivity, *Anal Chem* **82** (2010), 1027–1038.
- [28] O. Kooistra, E. Luneberg, B. Lindner, Y.A. Knirel, M. Frosch and U. Zahringer, Complex O-acetylation in *Legionella pneumophila* serogroup 1 lipopolysaccharide, evidence for two genes involved in 8-O-acetylation of legionaminic acid, *Biochemistry* **40** (2001), 7630–7640.
- [29] L.H. Krojgaard, K.A. Krogfelt, H.J. Albrechtsen and S.A. Uldum, Detection of *Legionella* by quantitative-polymerase chain reaction (qPCR) for monitoring and risk assessment, *BMC Microbiol* **11** (2011), 254.
- [30] J. Kügler, S. Wilke, D. Meier, F. Tomszak, A. Frenzel, T. Schirrmann, S. Dubel, H. Garritsen, B. Hock, L. Toleikis, M. Schutte and M. Hust, Generation and analysis of the improved human HAL9/10 antibody phage display libraries, *BMC Biotechnol* **15** (2015), 10.
- [31] P. Kuhn, V. Fuhner, T. Unkauf, G.M. Moreira, A. Frenzel, S. Miethel and M. Hust, Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display, *Proteomics Clin Appl* (2016).
- [32] X. Li, D. Lu, Z. Sheng, K. Chen, X. Guo, M. Jin and H. Han, A fast and sensitive immunoassay of avian influenza virus based on label-free quantum dot probe and lateral flow test strip, *Talanta* **100** (2012), 1–6.
- [33] C.E. Lucas, T.H. Taylor, Jr. and B.S. Fields, Accuracy and precision of *Legionella* isolation by US laboratories in the ELITE program pilot study, *Water Res* **45** (2011), 4428–4436.
- [34] P.O. Magalhaes, A.M. Lopes, P.G. Mazzola, C. Rangel-Yagui, T.C. Penna and A. Pessoa, Jr., Methods of endotoxin removal from biological preparations: a review, *J Pharm Pharm Sci* **10** (2007), 388–404.
- [35] E. Mauriz, A. Calle, J.J. Manclus, A. Montoya, A. Hildebrandt, D. Barcelo and L.M. Lechuga, Optical immunosensor for fast and sensitive detection of DDT and related compounds in river water samples, *Biosens Bioelectron* **22** (2007), 1410–1418.
- [36] B.J. McCarthy and A.S. Hill, Altering the fine specificity of an anti-*Legionella* single chain antibody by a single amino acid insertion, *J Immunol Methods* **251** (2001), 137–149.
- [37] G.K. Morris, C.M. Patton, J.C. Feeley, S.E. Johnson, G. Gorman, W.T. Martin, P. Skaliy, G.F. Mallison, B.D. Politi and D.C. Mackel, Isolation of the Legionnaires' disease bacterium from environmental samples, *Ann Intern Med* **90** (1979), 664–666.
- [38] H. Nikaido, Outer membrane, *Escherichia Coli and Salmonella* (1996), 29–47.
- [39] F.S. Nolte, C.A. Conlin and M.A. Motley, Electrophoretic and serological characterization of the lipopolysaccharides of *Legionella pneumophila*, *Infect Immun* **52** (1986), 676–681.
- [40] M. Palusinska-Szys and M. Cendrowska-Pinkosz, Pathogenicity of the family Legionellaceae, *Arch Immunol Ther Exp (Warsz)* **57** (2009), 279–290.
- [41] L. Pei, M. Rieger, S. Lengger, S. Ott, C. Zawadsky, N.M. Hartmann, H.C. Selinka, A. Tiehm, R. Niessner and M. Seidel, Combination of crossflow ultrafiltration, monolithic affinity filtration, and quantitative reverse transcriptase PCR for rapid concentration and quantification of model viruses in water, *Environ Sci Technol* **46** (2012), 10073–10080.
- [42] F. Petitjean, J.G. Guillet, B. Vray, J. Hoebeke, C. Tram and A.D. Strosberg, Standardization for diagnostic purposes of a monoclonal antibody against *Legionella pneumophila*, *Dev Biol Stand* **57** (1984), 99–105.
- [43] U. Reidt, B. Geisberger, C. Heller and A. Friedberger, Automated immunomagnetic processing and separation of *Legionella pneumophila* with manual detection by sandwich

- ELISA and PCR amplification of the ompS gene, *J Lab Autom* **16** (2011), 157–164.
- [44] Robert-Koch-Institut, Legionärskrankheit in Deutschland (2001 bis 2013), in: *Epidemiologisches Bulletin*, Nr 13, 2015.
- [45] T.J. Rowbotham, Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae, *J Clin Pathol* **33** (1980), 1179–1183.
- [46] J.O. Tobin, J. Beare, M.S. Dunnill, S. Fisher-Hoch, M. French, R.G. Mitchell, P.J. Morris and M.F. Muers, Legionnaires' disease in a transplant unit: Isolation of the causative agent from shower baths, *Lancet* **2** (1980), 118–121.
- [47] H. von Baum, S. Ewig, R. Marre, N. Suttorp, S. Gonschior, T. Welte, C. Luck and G. Competence, Network for community acquired pneumonia study, community-acquired legionella pneumonia: New insights from the German competence network for community acquired pneumonia, *Clin Infect Dis* **46** (2008), 1356–1364.
- [48] A. Wunderlich, C. Torggler, D. Elsasser, C. Luck, R. Niessner and M. Seidel, Rapid quantification method for *Legionella pneumophila* in surface water, *Anal Bioanal Chem* **408** (2016), 2203–2213.
- [49] V.L. Yu, J.F. Plouffe, M.C. Pastoris, J.E. Stout, M. Schousboe, A. Widmer, J. Summersgill, T. File, C.M. Heath, D.L. Patterson and A. Cheresky, Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: An international collaborative survey, *J Infect Dis* **186** (2002), 127–128.