Antimicrobial agent susceptibilities of *Legionella pneumophila* MLVA-8 genotypes

Yehonatan Sharaby1, Orna Nitzan2,3, Ingrid Brettar4, Manfred G. Höfle4, Avi Peretz2,3 & Malka Halpern1,5

*Legionella pneumophila* causes human lung infections resulting in severe pneumonia. High-resolution genotyping of *L. pneumophila* isolates can be achieved by multiple-locus variable-number tandem-repeat analysis (MLVA-8). *Legionella* infections in humans occur as a result of inhalation of bacteria-containing aerosols, thus, our aim was to study the antimicrobial susceptibilities of different MLVA-8 genotypes to ten commonly used antimicrobial agents in legionellosis therapy. Epidemiological cut-off values were determined for all antibiotics. Significant differences were found between the antimicrobial agents’ susceptibilities of the three studied environmental genotypes (Gt4, Gt6, and Gt15). Each genotype exhibited a significantly different susceptibility profile, with Gt4 strains (Sequence Type 1) significantly more resistant towards most studied antimicrobial agents. In contrast, Gt6 strains (also Sequence Type 1) were more susceptible to six of the ten studied antimicrobial agents compared to the other genotypes. Our findings show that environmental strains isolated from adjacent points of the same water system, exhibit distinct antimicrobial resistance profiles. These differences highlight the importance of susceptibility testing of *Legionella* strains. In Israel, the most extensively used macrolide for pneumonia is azithromycin. Our results point at the fact that clarithromycin (another macrolide) and trimethoprim with sulfamethoxazole (SXT) were the most effective antimicrobial agents towards *L. pneumophila* strains. Moreover, legionellosis can be caused by multiple *L. pneumophila* genotypes, thus, the treatment approach should be the use of combined antibiotic therapy. Further studies are needed to evaluate specific antimicrobial combinations for legionellosis therapy.

*Legionella pneumophila* has been found worldwide to be a relatively common pulmonary pathogen of severe community-acquired or nosocomial pneumonia2–9. *Legionella* infections in humans occur via inhalation of bacteria-containing aerosols, thus, the source of *Legionella* infection in humans is the environmental strains. Because of the ability of *Legionella* spp. to survive and multiply in human macrophages, they are susceptible to intracellularly active antimicrobial agents4–5. Currently, fluoroquinolones, macrolides, and rifampicin are the most commonly used antimicrobials in the treatment of legionellosis4,5. However, mortality rates of 10–15% are usually reported in legionellosis patients and death may occur despite antimicrobial agent therapy. Furthermore, the presence of antimicrobial agents in the environment may promote the evolution of microbial resistance mechanisms9. This is particularly important for *Legionella* spp. that colonize most man-made water systems, where they may be exposed to antimicrobial agents of various artificial origins, or even to those secreted by other microorganisms10.

Although 25 of the 59 described *Legionella* species have been implicated in human disease11–13, the vast majority of cases are caused by *L. pneumophila* strains, most of which belong to serogroup 114–16. Consequently, isolates of this common serogroup should be genotyped and further differentiated in order to evaluate the efficacy of antimicrobial agents in their treatment. Azithromycin is the most common macrolide used for treatment of...
abundances at their site of dominance. They could be addressed as different ecotypes with a distinct temperature range, growth kinetics, virulence and susceptibility profiles of different environmental and clinical legionella strains. It is important to assess the susceptibility patterns of legionella in order to get insights into ecological traits of strains inhabiting drinking water distribution systems (DWDSs). These studies showed that different sites of the same DWDS are dominated by different genotypes used in the current study. Overview of the studied genotypes and their MLVA-8 allelic profiles; number of tandem repeats observed for each L. pneumophila minisatellite locus (Lpms). The indicated sampling points in the drinking-water network were representative for the whole network. The water flow direction was from sampling point A to G. For more details regarding the sampling points please see Rodriguez-Martinez et al. Allelic repeats profiles for the reference strain were obtained from Pourcel et al. Highlighted in bold are differences in tandem repeats for each genotype compared to the type strain L. pneumophila Philadelphia-1.

| Sampling point | MLVA-8 Genotypes (n) | Sequence type (ST), Serogroup (Sg) | MLVA-8 genotype (Lpms) |
|----------------|----------------------|---------------------------------|-----------------------|
|                |                      |                                 | (1) (3) (13) (17) (19) (33) (34) (35) |
| Environmental strains |
| A              | Gt15 (11)            | NA, Sg3                         | 9 8 8 2 5 2 2 21      |
| B              | Gt4 (64)             | ST1, Sg1                        | 7 7 10 2 4 4 2 17    |
| C, D           | Gt6 (16)             | ST1, Sg1                        | 7 7 10 2 4 4 2 18    |
| D              | Gt18 (1)             | ST1, Sg1                        | 7 7 7 2 4 4 2 17    |
| E              | Gt3 (1)              | NA, Sg1                         | 7 7 10 2 4 4 2 0     |
| Clinical strains |
| Hospital       | Gt4 (4)              | ST1, Sg1                        | 7 7 10 2 4 4 2 17    |
| Hospital       | Gt6 (2)              | ST1, Sg1                        | 7 7 10 2 4 4 2 18    |
| Hospital       | Gt19 (1)             | ST1, Sg1                        | 7 7 10 1 4 4 2 17    |
| Hospital       | Gt20 (1)             | ST1, Sg1                        | 7 7 10 2 4 4 3 17    |
| Hospital       | Gt22 (2)             | ST59, Sg1                       | 8 8 10 2 5 4 1 13    |
| Hospital       | Gt24 (2)             | ST93, Sg1                       | 8 8 11 2 0 1 1 3     |
| Reference strain |
| Philadelphia-1 | Gt64                 | ST36, Sg1                       | 8 8 11 2 4 1 1 3     |

Table 1. Legionella pneumophila genotypes used in the current study. Overview of the studied genotypes and their MLVA-8 allelic profiles; number of tandem repeats observed for each L. pneumophila minisatellite locus (Lpms). The indicated sampling points in the drinking-water network were representative for the whole network. The water flow direction was from sampling point A to G. For more details regarding the sampling points please see Rodriguez-Martinez et al. Allelic repeats profiles for the reference strain were obtained from Pourcel et al. Highlighted in bold are differences in tandem repeats for each genotype compared to the type strain L. pneumophila Philadelphia-1.

community-acquired pneumonia in Israel. However, higher minimal inhibitory concentration (MIC) values have been reported for azithromycin compared with other macrolides for L. pneumophila serogroup 1. Thus, it is important to assess the susceptibility patterns of L. pneumophila in Israel as recommended in other countries.

Multiple-locus variable-number tandem-repeat analysis (MLVA) was implemented by Pourcel et al. and approved by the European Centre for Disease Prevention and Control. The method relies on the variability found in some tandemly repeated DNA sequences (VNTR) that represent sources of genetic polymorphism (Supplementary Fig. S1). This high-throughput typing method is used for epidemiological investigations of the origin of legionellosis cases since it allows rapid systematic typing of any new isolate and inclusion of data in shared databases.

Recently, Rodriguez-Martinez et al. and Sharaby et al. showed that the level of genotypes (analyzed by MLVA-8) should be addressed in order to get insights into ecological traits of L. pneumophila strains inhabiting drinking water distribution systems (DWDSs). These studies showed that different sites of the same DWDS are dominated by different L. pneumophila MLVA genotypes. Analysis of the three dominant genotypes showed that they could be addressed as different ecotypes with a distinct temperature range, growth kinetics, virulence and abundances at their site of dominance.

The aim of the current study was to analyze and compare the antimicrobial susceptibilities of different L. pneumophila genotypes to commonly used antimicrobial agents in legionellosis therapy. As far as we know, results from susceptibility testing of environmental and clinical L. pneumophila isolates have never been published in Israel. Since humans are infected with Legionella by inhaling Legionella-contaminated water aerosols, it is important to study the resistances of environmental strains to antibacterial agents and not only the clinical isolates. We determined the antimicrobial susceptibility profile for different L. pneumophila MLVA-8 genotypes. As each pneumonia patient can be infected by a mixture of L. pneumophila strains, studying the antimicrobial susceptibility profiles of different environmental and clinical L. pneumophila strains is of great importance as it may shed light on the distribution of resistance to antimicrobial agents and assist in determining an accurate and efficient treatment for future legionellosis patients.

Methods

L. pneumophila strains. We studied the susceptibility of 93 environmental and 12 clinical strains to 10 antimicrobial compounds that are commonly used for legionellosis (Table 1). These strains were isolated from a drinking-water distribution system (DWDS) as part of a study conducted in northern Israel for two years (2013–2014, between coordinates 32°42′N, 35°6′28.666″E). During the sampling campaign, we sampled Legionella spp. seasonally from the drinking water systems of seven buildings. Legionella was isolated from water and biofilm samples according to ISO 11731:2004 and 11731:2017 as described by Rodriguez-Martinez et al. In addition, we studied the susceptibility to antimicrobial agents of twelve clinical strains that were isolated from sputum samples of hospitalized pneumonia patients at Poriya and Rambam hospitals in northern Israel, between April 2013 and September 2014.
Reference strains.  *L. pneumophila* subsp. 1 of the American Type Culture Collection (ATCC 33152) was used as the reference strain. In addition,  *Staphylococcus aureus* (ATCC 29213) and  *Escherichia coli* (ATCC 25922) were also selected for validation of susceptibility testing results (Table 2). The selected strains were kept frozen at −80 °C prior to analysis.

**L. pneumophila** molecular typing. Genotyping of the strains was achieved by Multi Locus Variable number of tandem repeat (MLV A-8) analysis as described by Pourcel *et al.*21,22, Kahlisch *et al.*29 and Pecellin30 (Fig. 1). Briefly, 1 × 10^−2 ng of DNA template was used in 25 µl PCR reactions containing 1 Multiplex PCR Master Mix (Qiagen, Hilden, Germany) and 1.25 pmol of each primer (VIC®, NED®, FAM-, and NET-labeled forward primers from Applied Biosystems, Foster City, CA). Primers sequences, position, and repeat sizes at each variable number of tandem repeats (VNTR) locus are listed in Supplementary Table S1. After amplification, PCR products were pooled and denatured. Amplicons were then separated by size using fluorescent capillary electrophoresis, a powerful separation technique based on the differential size-dependent migration of DNA molecules in an electric field. Fluorescent capillary electrophoresis of the multiplex PCR products was performed with a 3730 × L sequencer (Applied Biosystems) as described in Nederbragt *et al.*21. We used a pre-run voltage of 8.0 kV, run voltage of 8 kV, injection voltage of 1.8 kV and injection time of 15 sec. Each *L. pneumophila* mini-microsatellite locus (Lpms) was identified by color and assigned a size by GeneMapper software, version 3.7 (Applied Biosystems). Repeat profiles were then compared with the MLV-8 database for *Legionella* ([http://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/887](http://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/887)). The workflow chart of the MLV-8 analysis is explained in Supplementary Fig. S1 and in the Methods section 2.3.

Table 2. Minimal inhibitory concentrations (µg/ml) of each antimicrobial agent, towards the reference strains. SXT, Trimethoprim and sulfamethoxazole; N.D., not determined.

| Antimicrobial Agent | L. pneumophila (ATCC 33152) | E. coli (ATCC 25922) | S. aureus (ATCC 29213) |
|---------------------|-----------------------------|----------------------|-----------------------|
| Ciprofloxacin       | 0.032                       | 0.25                 | N.D.                  |
| Moxifloxacin        | 0.032                       | 0.25                 | N.D.                  |
| Levofloxacin        | 0.032                       | 0.064                | 0.25                  |
| Tigecycline         | 0.064                       | 0.25                 | N.D.                  |
| Doxycycline         | 0.032                       | N.D.                 | 0.023                 |
| Azithromycin        | 0.032                       | N.D.                 | 0.023                 |
| Erythromycin        | 0.047                       | N.D.                 | 0.25                  |
| Clarithromycin      | 0.047                       | N.D.                 | 0.032                 |
| Rifampicin          | 0.023                       | N.D.                 | 0.25                  |
| Ciprofloxacin SXTa  | 0.023                       | 0.19                 | 2.0                   |

Figure 1. Representative electropherograms of MLVA-8 PCR products of multiplex PCR, separated by capillary electrophoresis and identified according to their sizes and colors. Electrophorograms correspond to MLVA-8 PCR products of panel 1 and panel 2 of (A) Genotype 64 (Gt64) *L. pneumophila* Philadelphia-1 and (B) Genotype 4 (Gt4) an environmental strain. Repeats number at each Lpms locus was identified by color and peak size by GeneMapper software, version 3.7 (Applied Biosystems). Repeat profiles were then compared with the MLVA-8 database for *Legionella* ([http://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/887](http://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/887)). The workflow chart of the MLVA-8 analysis is explained in Supplementary Fig. S1 and in the Methods section 2.3.
The MICs were read after 48 hours of incubation at 35 °C. Clarithromycin, Ciprofloxacin, Moxifloxacin, Rifampicin, Tigecycline, Doxycycline, Levofloxacin, Erythromycin, and Trimethoprim-sulfamethoxazole. The MICs were read after 48 hours of incubation at 35 °C at 2.5% CO₂. The MIC of each antimicrobial agent was taken as the lowest concentration of the antimicrobial agent at which the zone of inhibition intersected the strip. MIC tests for *L. pneumophila* isolates were repeated in triplicates. Epidemiological Cut-off values (ECOFFs) were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for *Legionella pneumophila*. Briefly, MIC values were fitted to the cumulative log-normal distribution using non-linear least squares regression in order to determine the ECOFF for each antimicrobial agent.

**Statistical analysis.** All statistical analyses were performed using IBM SPSS 22® and Primer7 software (Primer-e, Auckland, New Zealand). All tests were applied at a 95% level of confidence. Repeated-measures analysis of variance (ANOVA) was applied to study the differences between the MICs of different antimicrobials. Data sphericity was not violated (Mauchly's test: *p* > 0.05). T-tests were applied in order to compare the antimicrobials' MICs for strains isolated from environmental versus clinical sources, water versus biofilm and hot versus cold water. In addition, analysis of similarities (ANOSIM) was performed in order to compare the antimicrobial agent resistance profiles of environmental genotypes and clinical isolates taking into account all studied antimicrobial agents' MIC values. The resemblance matrix was calculated using the Bray-Curtis index of association (Primer7 software). One-way ANOVA was used to determine whether significant differences exist in antimicrobial agents' MICs between different MLVA-8 genotypes (Gt4, Gt6, and Gt15). All groups were normally distributed according to Shapiro-Wilk test (*p* > 0.05) and variances were equal between groups (Levene's test: *p* > 0.05).

**Results**

The susceptibilities of 93 environmental and 12 clinical *L. pneumophila* strains to 10 antimicrobial agents commonly used in legionellosis therapy were analyzed. The environmental strains that were studied here represent a subset of the strains belonging to three MLVA-8 genotypes (Gt) 4, 6, and 15 that dominated a water network in northern Israel (Table 1). The clinical strains belonged to Gt4 and Gt6, Gt19, Gt20, Gt22, and Gt24 (Table 1). All strains except Gt15 were classified as serogroup 1. Gt15 strains were classified as serogroup 3 (Table 1). We used *L. pneumophila* subsp. *Pneumophila* sg 1 (ATCC 33152) as the reference strain. In addition, *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were also selected for validation of susceptibility testing results (Table 2). The MICs obtained for both *S. aureus* and *E. coli* were generally lower but within an order of magnitude compared to the findings of previous studies.

Overall, significant differences were observed in *L. pneumophila* sensitivities to different antimicrobial agents (Repeated measures ANOVA: *F* 15.27, *p* < 0.001). Minimal inhibitory concentrations (MICs) of antimicrobial agents from the fluoroquinolone family were significantly higher compared to those of the macrolides, doxycycline, rifampicin, and trimethoprim & sulfamethoxazole (SXT) (Fig. 2, Table 3). The lowest MICs were observed after exposure to SXT, yet no significant differences were found between the MICs of SXT, erythromycin, Clarithromycin, and Rifampicin (Fig. 2). The highest MIC was found for ciprofloxacin (0.74 ± 0.06 µg/ml) and it was significantly higher than the MICs of moxifloxacin and levofloxacin, which are fluoroquinolones (0.52 ± 0.04 and 0.37 ± 0.04 µg/ml, respectively). MIC₅₀ values yielded similar results with the highest MIC₅₀ found for ciprofloxacin (0.75 µg/ml) and the lowest for SXT with 0.023 µg/ml (Tables 3, 4).

No significant differences were detected between the susceptibilities of environmental strains isolated from bulk water (*n* = 58) vs. biofilms (*n* = 35) (t-tests: *df* = 91, *p* > 0.1, for all studied antimicrobial agents). Moreover, t-tests did not detect any significant differences in antimicrobial agent resistances of strains isolated from cold (*n* = 32) vs. hot water (*n* = 26) (t-tests: *df* = 56, *p* > 0.1, for all studied antimicrobial agents). For additional details, see Supplemental Table S2. In contrast, t-tests revealed significant differences in antimicrobial agent susceptibilities of environmental vs. clinical (e.g., isolated from patients’ sputum) strains. Environmental strains were significantly more resistant towards five of the 10 studied antimicrobial agents compared to *L. pneumophila* strains from clinical sources (Table 5). The largest difference was found after exposure to ciprofloxacin; MIC₅₀ of ciprofloxacin was 1.0 µg/ml for the environmental strains and only 0.22 µg/ml for the clinical strains (Table 5). In addition, the MICs of tigecycline, clarithromycin, rifampicin, and SXT were also significantly higher for the environmental strains compared to the clinical *L. pneumophila* strains (Table 5). In contrast, doxycycline was the only studied antimicrobial agent for which the clinical strains were more resistant, with MIC₅₀ and MIC₉₀ of 0.19 and 0.5 µg/ml compared to 0.25 and 0.032 µg/ml for the environmental strains, respectively (Table 5). In addition, analysis of similarities (ANOSIM) revealed significant differences between the antimicrobial agent resistance profiles of clinical and environmental *L. pneumophila* isolates (*R* = 0.62, *p* < 0.001). However, a comparison of Gt4 strains from...
Figure 2. Minimal inhibitory concentrations (average ± standard error) of each studied antimicrobial agent towards *L. pneumophila* strains isolated from both clinical and environmental sources (*n* = 105). Ciprofloxacin – CIP, moxifloxacin – MXF, levofloxacin – LEV, tigecycline – TGC, doxycycline – DXT, azithromycin – AMZ, erythromycin – E, clarithromycin – CLR, rifampicin – RD, trimethoprim & sulfamethoxazole – SXT. Bars connected by different letters are significantly different by repeated-measures ANOVA with Tukey’s HSD post-hoc test with a confidence interval of 95%.

Table 3. The accumulated percentages (%) of all the tested strains (93 environmental and 12 clinical *L. pneumophila* isolates), that were inhibited at each concentration of the different antimicrobial agents (µg/ml). *SXT, Trimethoprim and sulfamethoxazole. The values for each antimicrobial agent are the percentage of the strains that were inhibited in the above mentioned antimicrobial agent concentration, thus, MIC<sub>50</sub> and MIC<sub>90</sub> values can be read directly from this table. For example, SXT has a MIC<sub>50</sub> of between 0.016–0.023 µg/ml and a MIC<sub>90</sub> of 0.25 µg/ml.

| Drug              | MIC<sub>50</sub> | MIC<sub>90</sub> | Range    | ECOFF* |
|-------------------|-----------------|-----------------|----------|--------|
| Ciprofloxacin     | 0.75            | 1.5             | 0.019–2.0| 4.0    |
| Moxifloxacin      | 0.5             | 1.0             | 0.032–1.5| 4.0    |
| Levofloxacin      | 0.075           | 1.0             | 0.023–1.5| 4.0    |
| Tigecycline       | 0.5             | 1.5             | 0.023–2.0| 0.5    |
| Doxycycline       | 0.032           | 0.5             | 0.023–0.5| 0.5    |
| Azithromycin      | 0.38            | 0.75            | 0.032–1.0| 2.0    |
| Erythromycin      | 0.094           | 0.5             | 0.023–1.0| 0.5    |
| Clarithromycin    | 0.064           | 0.25            | 0.025–0.5| 0.5    |
| Rifampicin        | 0.023           | 0.5             | 0.003–1.0| 0.063  |
| SXT**             | 0.023           | 0.25            | 0.003–0.75| 0.5  |

Table 4. MIC<sub>50</sub>, MIC<sub>90</sub>, MIC range and ECOFF values (µg/ml) of the 10 tested antimicrobial agents for all *L. pneumophila* strains (*n* = 105). MIC<sub>50</sub>, MIC<sub>90</sub>. Lowest concentration of the antimicrobial agents at which 50% and 90% of the isolates were inhibited, respectively. *ECOFF, epidemiological cut-off values. **SXT, Trimethoprim and sulfamethoxazole.
Table 5. Antimicrobial MICs (µg/ml) for the clinical and the environmental L. pneumophila strains and for each of the environmental genotypes. Environmental strains included two strains designated Gt3 and Gt18 in addition to the listed Gt4, Gt6, Gt15 strains. **SXT, Trimethoprim and sulfamethoxazole. MIC90 values are in bold and MIC50 values are presented in brackets.

|                  | Gt4 (n = 64) | Gt6 (n = 16) | Gt15 (n = 11) | Environmental (n = 93) | Clinical (n = 12) |
|------------------|--------------|--------------|---------------|------------------------|-------------------|
| Ciprofloxacin    | 2.0 (1)      | 0.875 (0.22) | 1.0 (0.5)     | 1.5 (1)                | 0.475 (0.22)      |
| Moxifloxacin     | 1.0 (0.75)   | 0.25 (0.0395)| 1.0 (0.5)     | 1.0 (0.5)              | 1.0 (0.5)         |
| Levofloxacin     | 1.0 (0.5)    | 0.157 (0.032)| 0.75 (0.032)  | 1.0 (0.064)            | 0.75 (0.238)      |
| Tigecycline      | 1.5 (0.75)   | 0.056 (0.032)| 0.5 (0.047)   | 1.5 (0.5)              | 0.5 (0.047)       |
| Doxycycline      | 0.25 (0.032) | 0.19 (0.032) | 0.5 (0.064)   | 0.25 (0.032)           | 0.5 (0.19)        |
| Azithromycin     | 0.75 (0.44)  | 0.19 (0.0555)| 0.5 (0.25)    | 0.75 (0.38)            | 0.725 (0.375)     |
| Erythromycin     | 0.5 (0.079)  | 0.25 (0.142) | 0.5 (0.19)    | 0.5 (0.125)            | 0.19 (0.032)      |
| Clarithromycin   | 0.25 (0.056) | 0.5 (0.064)  | 0.5 (0.25)    | 0.25 (0.032)           | 0.64 (0.047)      |
| Rifampicin       | 0.05 (0.032) | 0.012 (0.006)| 1.0 (0.032)   | 0.5 (0.032)            | 0.006 (0.004)     |
| SXT**            | 0.25 (0.023) | 0.253 (0.032)| 0.5 (0.032)   | 0.354 (0.032)          | 0.023 (0.006)     |

Figure 3. Minimal inhibitory concentrations (average ± standard error) of each studied antimicrobial agent for different MLVA-8 genotypes. Ciprofloxacin – CIP, moxifloxacin – MXF, levofloxacin – LEV, tigecycline – TGC, doxycycline – DXT, azithromycin – AMZ, erythromycin – E, clarithromycin – CLR, rifampicin – RD, trimethoprim & sulfamethoxazole – SXT. Asterisks represent significant differences by one-way ANOVA with Tukey’s post-hoc tests between genotypes at the 0.05* and 0.001** levels of confidence. n.s., not significant.

**Environmental genotypes.** One-way ANOVA revealed significant differences in the resistance of the co-localized environmental genotypes (F2,66 = 128.73, p < 0.001). Gt4 strains were found to be significantly more resistant towards ciprofloxacin, moxifloxacin, levofloxacin, tigecycline, and azithromycin compared to strains belonging to Gt6 and Gt15 (Fig. 3 and Table 5). The highest MIC90 values were obtained for Gt4 strains after exposure to ciprofloxacin and tigecycline (2 µg/ml and 1.5 µg/ml, respectively). The MIC50 values of Gt6 strains were significantly lower compared to other genotypes after exposure to six out of the ten studied antimicrobial agents. The lowest MIC90 values for Gt6 strains were obtained with tigecycline and rifampicin (0.056 and 0.012 µg/ml, respectively); an order of magnitude lower compared to the MICs of Gt4 and Gt15 strains (Fig. 3 and Table 5). Gt15 strains were significantly more resistant to clarithromycin, rifampicin, and SXT (with MIC90 of 0.5, 1, and 0.5 µg/ml, respectively). In addition, analysis of similarities showed that different genotypes possess significantly different resistance profiles (ANOSIM: R = 0.287, p = 0.001).

**Discussion.** MLVA is a useful genotyping method as it allows a good resolution within the highly health-relevant and abundant Sequence Type 1 (ST1) strains (Table 1). For example, genotypes 4 and 6 are both classified as ST1, and cannot be differentiated by the sequence-based typing method. Moreover, genotype 4 comprises the reference strain L. pneumophila Paris, which belongs to ST119. Mercante and Winchell12 and McDade35 have suggested that the level of genotypes should be addressed in order to assess the health risks posed by the presence of different L. pneumophila strains in DWDSs. As far as we know this is the first study that compares susceptibilities of environmental L. pneumophila MLVA-8 genotypes to antimicrobial agents.

Recently, we have demonstrated that L. pneumophila dominated different sites of a small Israeli drinking water network, with MLVA-8 genotype related abundance regime22. These genotypes demonstrated different
temperature-dependent growth kinetics and different cytotoxicity towards amoebae, macrophages and red blood cells. Hence, here we show that these same isolates differed also in their susceptibilities to antimicrobial agents (Tables 1 and 5). MLVA-8 genotypes 4 and 6 strains exhibited distinct growth characteristics despite the fact that both are classified as ST1 by sequence-based typing. Gt4 strains were able to proliferate more rapidly in temperatures of 25–37 °C compared to genotypes Gt6 and Gt15 strains. In addition, Gt4 strains were significantly more cytotoxic towards amoebae and macrophages under *in vitro* experimental conditions. In the current study, Gt4 strains were significantly more resistant towards five out of the 10 antimicrobial agents that were studied, compared to Gt6 strains (Fig. 3). These findings suggest that ST1 strains belonging to Gt4 genotypes may pose a much more severe health risk compared to ST1 strains belonging to Gt6 (Tables 1 and 5). Our current findings indicate that these environmental genotypes, although colonizing the same niche in the drinking water system, should be addressed as different ecotypes since a high variability exists even among ST1 strains in terms of their antimicrobial resistance profiles.

Coscollà et al. observed mixed infections of *L. pneumophila* strains in outbreak patients. They analyzed sequence based typing profiles of uncultured respiratory samples and found evidence of a mixture of *Legionella* ST profiles in patients. They concluded that patients might be infected from the environment by more than one *L. pneumophila* strain. Recently, Mizrahi et al. also reported that a mix of *L. pneumophila* strains were identified from sputum samples of pneumonia patients. These findings, along with the results described here regarding the high variability of *L. pneumophila* genotypes’ antimicrobial agent resistances, emphasize the importance of high-resolution identification of different genotypes and their antimicrobial agent susceptibility profiles, especially in pneumonia patients. In such cases of mixed lung infections caused by multiple *L. pneumophila* genotypes, the application of combination of antibiotic therapy should be considered since it might provide better treatment outcomes.

Dual combination antibiotic therapy was shown to improve treatment outcomes and survival in patients with severe community-acquired pneumonia caused by *Legionella* and other pathogenic bacteria. Adding a macrolide or fluoroquinolone to a *β-lactam* was already recommended by the Infectious Diseases Society of America/American Thoracic Society guidelines. For example, the combination of rifampicin with clarithromycin showed decreased mortality rates in patients. In our study, both rifampicin and clarithromycin, were found to be very effective towards the three compared genotypes (Fig. 3 and Table 5). Therefore, their combination in treating mixed infections caused by several *L. pneumophila* genotypes may improve treatment outcomes compared to monotherapy. Further research with emphasis on different MLVA genotyping will allow more accurate assessments of the different antimicrobials’ efficacies in treatment of human infections.

It has been previously reported that performing E-test on BCYE-α agar may yield elevated MICs. Nonetheless, it still provides a simple yet accurate method for routine and comparative susceptibility testing of *Legionella* spp. However, the MIC value itself, should not be directly translated to serum concentrations of these antimicrobial agents. Thus, it can be used for detecting antimicrobial resistances. Sufficient data to establish ECOFFs are currently not available. In the current study, ECOFFs were determined according to the EUCAST guidelines for *L. pneumophila* susceptibility testing (Table 4). Our findings can be used in the future in the process of setting epidemiological cut off values.

Antimicrobial agent susceptibility of *Legionella* strains isolated from drinking water sources was studied previously. Xiong et al. found that levofloxacin was the most effective drug against different *L. pneumophila* serogroups. Minocycline and doxycycline were also found to be effective. Torre et al. and Sikora et al. found that ciprofloxacin and rifampicin have good activity against environmental *L. pneumophila* sg 1 and sg 2–14. For the overall set of strains tested in the current study, we found that the most effective drugs towards *L. pneumophila* strains were doxycycline, clarithromycin, rifampicin, and SXT (Fig. 2). Moreover, the strains in the current study were found to be relatively resistant towards levofloxacin and ciprofloxacin (the most effective drugs according to Xiong et al. and Sikora et al., respectively).

Azithromycin (macrolides) and respiratory fluoroquinolones are the most commonly used antimicrobial agent treatments for community-acquired pneumonia. Numerous public health agencies such as the Infectious Diseases Society of America (IDSA), the British Thoracic Society (BTS) and the Dutch Association of Chest Physicians recommend using fluoroquinolones (ciprofloxacin in particular), or azithromycin, as a preferred antimicrobial therapy for legionellosis cases. Thus, it is of major importance to verify high susceptibility rates of *L. pneumophila* to these antimicrobial agents. We found significantly higher MIC values to fluoroquinolones compared with macrolides, which might justify empiric and definitive treatment with macrolides as first line treatment of *L. pneumophila* pneumonia in Israel (Fig. 2, Table 3). In contrast, other studies reported that quinolones have greater activity toward rate assessments of the different antimicrobials’ efficacies in treatment of human infections.

High variability of *L. pneumophila* genotypes’ antimicrobial agent resistances, emphasize the importance of high-resolution identification of different genotypes and their antimicrobial agent susceptibility profiles, especially in pneumonia patients. In such cases of mixed lung infections caused by multiple *L. pneumophila* genotypes, the application of combination of antibiotic therapy should be considered since it might provide better treatment outcomes. Dual combination antibiotic therapy was shown to improve treatment outcomes and survival in patients with severe community-acquired pneumonia caused by *Legionella* and other pathogenic bacteria. Adding a macrolide or fluoroquinolone to a *β-lactam* was already recommended by the Infectious Diseases Society of America/American Thoracic Society guidelines. For example, the combination of rifampicin with clarithromycin showed decreased mortality rates in patients. In our study, both rifampicin and clarithromycin were found to be very effective towards the three compared genotypes (Fig. 3 and Table 5). Therefore, their combination in treating mixed infections caused by several *L. pneumophila* genotypes may improve treatment outcomes compared to monotherapy. Further research with emphasis on different MLVA genotyping will allow more accurate assessments of the different antimicrobials’ efficacies in treatment of human infections.

It has been previously reported that performing E-test on BCYE-α agar may yield elevated MICs. Nonetheless, it still provides a simple yet accurate method for routine and comparative susceptibility testing of *Legionella* spp. However, the MIC value itself, should not be directly translated to serum concentrations of these antimicrobial agents. Thus, it can be used for detecting antimicrobial resistances. Sufficient data to establish ECOFFs are currently not available. In the current study, ECOFFs were determined according to the EUCAST guidelines for *L. pneumophila* susceptibility testing (Table 4). Our findings can be used in the future in the process of setting epidemiological cut off values.

Antimicrobial agent susceptibility of *Legionella* strains isolated from drinking water sources was studied previously. Xiong et al. found that levofloxacin was the most effective drug against different *L. pneumophila* serogroups. Minocycline and doxycycline were also found to be effective. Torre et al. and Sikora et al. found that ciprofloxacin and rifampicin have good activity against environmental *L. pneumophila* sg 1 and sg 2–14. For the overall set of strains tested in the current study, we found that the most effective drugs towards *L. pneumophila* strains were doxycycline, clarithromycin, rifampicin, and SXT (Fig. 2). Moreover, the strains in the current study were found to be relatively resistant towards levofloxacin and ciprofloxacin (the most effective drugs according to Xiong et al. and Sikora et al., respectively).

Azithromycin (macrolides) and respiratory fluoroquinolones are the most commonly used antimicrobial agent treatments for community-acquired pneumonia. Numerous public health agencies such as the Infectious Diseases Society of America (IDSA), the British Thoracic Society (BTS) and the Dutch Association of Chest Physicians recommend using fluoroquinolones (ciprofloxacin in particular), or azithromycin, as a preferred antimicrobial therapy for legionellosis cases. Thus, it is of major importance to verify high susceptibility rates of *L. pneumophila* to these antimicrobial agents. We found significantly higher MIC values to fluoroquinolones compared with macrolides, which might justify empiric and definitive treatment with macrolides as first line treatment of *L. pneumophila* pneumonia in Israel (Fig. 2, Table 3). In contrast, other studies reported that quinolones have greater activity toward rate assessments of the different antimicrobials’ efficacies in treatment of human infections.

High variability of *L. pneumophila* genotypes’ antimicrobial agent resistances, emphasize the importance of high-resolution identification of different genotypes and their antimicrobial agent susceptibility profiles, especially in pneumonia patients. In such cases of mixed lung infections caused by multiple *L. pneumophila* genotypes, the application of combination of antibiotic therapy should be considered since it might provide better treatment outcomes. Dual combination antibiotic therapy was shown to improve treatment outcomes and survival in patients with severe community-acquired pneumonia caused by *Legionella* and other pathogenic bacteria. Adding a macrolide or fluoroquinolone to a *β-lactam* was already recommended by the Infectious Diseases Society of America/American Thoracic Society guidelines. For example, the combination of rifampicin with clarithromycin showed decreased mortality rates in patients. In our study, both rifampicin and clarithromycin were found to be very effective towards the three compared genotypes (Fig. 3 and Table 5). Therefore, their combination in treating mixed infections caused by several *L. pneumophila* genotypes may improve treatment outcomes compared to monotherapy. Further research with emphasis on different MLVA genotyping will allow more accurate assessments of the different antimicrobials’ efficacies in treatment of human infections.

It has been previously reported that performing E-test on BCYE-α agar may yield elevated MICs. Nonetheless, it still provides a simple yet accurate method for routine and comparative susceptibility testing of *Legionella* spp. However, the MIC value itself, should not be directly translated to serum concentrations of these antimicrobial agents. Thus, it can be used for detecting antimicrobial resistances. Sufficient data to establish ECOFFs are currently not available. In the current study, ECOFFs were determined according to the EUCAST guidelines for *L. pneumophila* susceptibility testing (Table 4). Our findings can be used in the future in the process of setting epidemiological cut off values.

Antimicrobial agent susceptibility of *Legionella* strains isolated from drinking water sources was studied previously. Xiong et al. found that levofloxacin was the most effective drug against different *L. pneumophila* serogroups. Minocycline and doxycycline were also found to be effective. Torre et al. and Sikora et al. found that ciprofloxacin and rifampicin have good activity against environmental *L. pneumophila* sg 1 and sg 2–14. For the overall set of strains tested in the current study, we found that the most effective drugs towards *L. pneumophila* strains were doxycycline, clarithromycin, rifampicin, and SXT (Fig. 2). Moreover, the strains in the current study were found to be relatively resistant towards levofloxacin and ciprofloxacin (the most effective drugs according to Xiong et al. and Sikora et al., respectively).

Azithromycin (macrolides) and respiratory fluoroquinolones are the most commonly used antimicrobial agent treatments for community-acquired pneumonia. Numerous public health agencies such as the Infectious Diseases Society of America (IDSA), the British Thoracic Society (BTS) and the Dutch Association of Chest Physicians recommend using fluoroquinolones (ciprofloxacin in particular), or azithromycin, as a preferred antimicrobial therapy for legionellosis cases. Thus, it is of major importance to verify high susceptibility rates of *L. pneumophila* to these antimicrobial agents. We found significantly higher MIC values to fluoroquinolones compared with macrolides, which might justify empiric and definitive treatment with macrolides as first line treatment of *L. pneumophila* pneumonia in Israel (Fig. 2, Table 3). In contrast, other studies reported that quinolones have greater activity toward rate assessments of the different antimicrobials’ efficacies in treatment of human infections.
Legionella patients are infected with the bacteria by inhaling water droplets containing Legionella. Thus, the source of the clinical strains is the environmental strains and it is important and useful to predict the onset of antimicrobial agent resistance in the environment before it is evidenced in clinical specimens. In the current study, environmental strains were significantly more resistant towards five (ciprofloxacin, tigecycline, clarithromycin, rifampicin, and SXT) out of the 10 studied antibacterial agents, compared to strains of clinical source. Clinical strains were significantly more resistant only to Doxycycline compared to the environmental strains (Table 5). In addition, the antimicrobial resistance profiles of clinical and environmental strains differed significantly (Table 5). Earlier studies suggested that the presence of antimicrobial agents in the environment, and especially in man-made drinking-water distribution systems (DWDSs), might promote the evolution of microbial resistance mechanisms.

Since only one case of person-to-person transmission of Legionella has been reported so far, the human body is considered to be a “dead-end” for the evolution of this pathogen. Therefore, clinical strains probably do not transfer antimicrobial resistances to the environment and the environmental strains are the source for the clinical cases of L. pneumophila pneumonia infections. It is of great importance to adjust the antibacterial therapy for legionellosis patients to fit the susceptibilities of environmental strains that are present in DWDSs. Our results show that a considerable amount of variability exists in terms of antimicrobial resistances of environmental strains (Fig. 3 and Table 5). A rapid and reliable method for distinguishing between strains is necessary in order to determine the specific susceptibilities of environmental L. pneumophila genotypes.

Routine monitoring and susceptibility testing of environmental strains from DWDSs can allow detection of antimicrobial resistances acquisition. However, as reported in previous studies, there are difficulties in determining MICs for Legionella (for example, inactivation of some antibiotics by charcoal). Consequently, it is difficult to compare results obtained from different methods and establish ECOFF values. Therefore, highly efficient techniques are needed in order to isolate environmental Legionella strains from the environment and then test and monitor the acquisition of resistance in the environmental context of the network.

In conclusion, we determined the antimicrobial agent susceptibility profiles for different L. pneumophila MLVA-8 genotypes. Gt4 strains belonging to ST1 were significantly more resistant towards Ciprofloxacin, Moxifloxacin, Levofloxacin, Tigecycline, and Azithromycin compared to strains belonging to Gt6 (also belonging to ST1), and Gt15 genotypes (Fig. 3). Our results demonstrate that although these environmental strains were isolated from adjacent points of the same drinking water system, they are distinct in terms of their antimicrobial agent susceptibilities as was also observed for their other physiological traits. Evidence pointed out that pneumonia patients may acquire a mixture of L. pneumophila strains. These, along with the results regarding the high variability of L. pneumophila genotypes’ antimicrobial resistance profiles, emphasize the importance of studying antimicrobial resistances of different L. pneumophila genotypes. Moreover, since the human body is considered a “dead-end” for the evolution of Legionella, it is important to study the antimicrobial resistances not only for clinical isolates, but also for the environmental strains that are the source of the clinical infections.

References
1. Adams, D. et al. Summary of Notifiable Infectious Diseases and Conditions — United States, 2013. MMWR Morb Mortal Wkly Rep 62, 1–119 (2013).
2. Lee, H. K., Shim, J. I., Kim, H. E., Yu, J. Y. & Kang, Y. H. Distribution of Legionella species from environmental water sources of public facilities and genetic diversity of L. pneumophila serogroup 1 in South Korea. Appl Environ Microbiol 76, 6547–54 (2010).
3. Qin, T. et al. Distribution of sequence-based types of Legionella pneumophila serogroup I strains isolated from cooling towers, hot springs, and potable water systems in China. Appl Environ Microbiol 80, 2150–2157 (2014).
4. Postma, D. F. et al. Antibiotic Treatment Strategies for Community-Acquired Pneumonia in Adults. N Engl J Med 372, 1312–1323 (2015).
5. Cunha, B. A., Burillo, A. & Bouza, E. Legionnaires’ disease. Lancet 387, 376–385 (2016).
6. Wiersinga, W. J. et al. Management of community-acquired pneumonia in adults: 2016 guideline update from the Dutch Working Party on Antibiotic Policy (SWAB) and Dutch Association of Chest Physicians (NVALT). Neth J Med 76, 4–13 (2018).
7. Edelstein, P. H. & Roy, C. R. Legionnaires’ Disease and Pontiac Fever. In Mandell, Douglas, and Bennett’s Principles and Practice of Infectious Diseases, https://doi.org/10.1016/B978-1-4557-4801-3.00234-4 (2014).
8. Kello, J. et al. Community-acquired Legionella Pneumonia in the intensive care unit: Impact on survival of combined antibiotic therapy. Med Intensiva, https://doi.org/10.1016/j.medint.2012.05.010 (2013).
9. D’Costa, V. M., McGrann, K. M., Hughes, D. W. & Wright, G. D. Sampling the Antibiotic Resistome. Science (80-) 311, 374–377 (2006).
10. Almahmoud, I., Kay, E., Schneider, D. & Maurin, M. Mutational paths towards increased fluoroquinolone resistance in Legionella pneumophila. J Antimicrob Chemother 64, 284–293 (2009).
11. Diederen, B. M. W. Legionella spp. and Legionnaires’ disease. J Infect 56, 1–12 (2008).
12. Mercante, J. W. & Winchell, J. M. Current and existing Legionella diagnostics for laboratory and outbreak investigations. Clin Microbiol Rev 28, 95–133 (2015).
13. Edelstein, P. H., Luck, C. & LUCK, C. Legionella. In Manual of Clinical Microbiology, 11th Edition 887–904 American Society of Microbiology, https://doi.org/10.1128/9780195581738_ch49 (2013).
14. Yu, Y. L. et al. Distribution of Legionella species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J Infect Dis 186, 127–128 (2002).
15. Cao, B., Yeo, F., Liu, X., Feng, L. & Wang, L. Development of a DNA microarray method for detection and identification of all 15 distinct O-antigen forms of Legionella pneumophila. Appl Environ Microbiol 79, 6647–54 (2013).
16. Marston, B. J. et al. Incidence of Community-Acquired Pneumonia Requiring Hospitalization. Results of a population-based active surveillance study in Ohio. Ann Intern Med 157, 1709–1718 (1997).
17. De Giglio, O. et al. Antibiotic susceptibility of Legionella pneumophila strains isolated from hospital water systems in Southern Italy. Environ Res 142, 586–590 (2015).
18. Pourcel, C., Vidgoy, Y., Ramisse, F., Vergnaud, G. & Tram, C. Characterization of a Tandem Repeat Polymorphism in Legionella pneumophila and Its Use for Genotyping. J Clin Microbiol 41, 1819–1826 (2003).
19. Pourcel, C. et al. Identiﬁcation of Variable-Number Tandem Repeat (VNTR) Sequences in Legionella pneumophila and Development of an Optimized Multiple-Locus VNTR Analysis Typing Scheme. J Clin Microbiol 45, 1190–1199 (2007).
20. Sobral, D. et al. High-Throughput Typing Method To Identify a Non-Outbreak-Involved Legionella pneumophila Strain Colonizing the Entire Water Supply System in the Town of Rennes, France. Appl Environ Microbiol 77, 6899–6907 (2011).
21. Nederbragt, A. J. et al. Multiple-locus variable-number tandem repeat analysis of Legionella pneumophila using multi-colored capillary electrophoresis. J Microbiol Methods 73, 111–17 (2008).

22. Rodríguez-Martínez, S. et al. Spatial distribution of Legionella pneumophila MLVA-genotypes in a drinking water system. Water Res 77, 119–132 (2015).

23. Sharaby, Y. et al. Temperature-Dependent Growth Modeling of Environmental and Clinical Legionella pneumophila Multilocus Variable-Number Tandem Repeat Analysis (MLVA) Genotypes. Appl Environ Microbiol 83, e03295–16 (2017).

24. Díaz del Alba, D. et al. Virulence Traits of Environmental and Clinical Legionella pneumophila Multilocus Variable-Number Tandem-Repeat Analysis (MLVA) Genotypes. Appl Environ Microbiol 84, AEM.00429–18 (2018).

25. Coscollà, M., Fernández, C., Colomina, J., Sánchez-Busó, L. & González-Candela, F. Mixed infection by Legionella pneumophila in outbreak patients. Int J Med Microbiol 304, 307–313 (2014).

26. Mizrahi, H. et al. Comparison of sputum microbiome of legionellosis-associated patients and other pneumonia patients: indications for polybacterial infections. Sci Rep 7 (2017).

27. International Organization for Standardization. ISO 11731-2:2004: water quality — detection and enumeration of Legionella — part 2: direct membrane filtration method for waters with low bacterial count (2004).

28. International Organization for Standardization. ISO 11731:2017 water quality — enumeration of Legionella (2017).

29. Kahlisch, L., Henne, K., Drahem, J., Brettar, I. & Höfle, M. G. High-resolution in situ genotyping of Legionella pneumophila populations in drinking water by multiple-locus variable-number tandem-repeat analysis using environmental DNA. Appl Environ Microbiol 76, 6186–90 (2010).

30. Pecellin, M. Structure and virulence of Legionella pneumophila populations from freshwater systems in Germany and Middle East. (Technical University of Braunschweig, Germany, 2016).

31. Tumridge, J., Kahlmeter, G. & Kronvall, G. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clinical Microbiology and Infection 12(5), 418–425 (2006).

32. Clarke, K. R. Non-parametric multivariate analyses of changes in community structure. Aust Ecol 18, 117–143 (1993).

33. Bruin, J. P., Ijzerman, E. P. F., den Boer, J. W., Mouton, J. W. & Diederken, B. M. W. Wild-type MIC distribution and epidemiological cut-off values in clinical Legionella pneumophila serogroup 1 isolates. Diagn Microbiol Infect Dis 72, 103–108 (2012).

34. Torre, I. et al. Environmental surveillance and in vitro activity of antimicrobial agents against Legionella pneumophila isolated from hospital water systems in Campania, South Italy: a 5-year study. Environ Res 164, 574–579 (2018).

35. McDade, J. E. Legionella and the Prevention of Legionellosis. Emerg Infect Dis 14, 1006a–1006 (2008).

36. Nie, W., Li, B. & Xiu, Q. β-Lactam/macrolide dual therapy versus β-lactam monotherapy for the treatment of community-acquired pneumonia in adults: a systematic review and meta-analysis. J Antimicrob Chemother 69, 1441–1446 (2014).

37. Mandell, L. A. et al. Infectious Diseases Society of America/American Thoracic Society Consensus Guidelines on the Management of Community-Acquired Pneumonia in Adults. Clin Infect Dis 62, 527–572 (2016).

38. Garcia, M. T., Pelaz, C., Gimenez, M. J. & Aguilar, L. In Vitro Activities of Gemifloxacin versus Five Quinolones and Two Macrolides against 271 Spanish Isolates of Legionella pneumophila: Influence of Charcoal on Susceptibility Test Results. Antimicrob Agents Chemother 44, 2176–2178 (2000).

39. Kahlisch, L., Henne, K., Drahem, J., Brettar, I. & Höfle, M. G. High-resolution in situ genotyping of Legionella pneumophila populations in drinking water by multiple-locus variable-number tandem-repeat analysis using environmental DNA. Appl Environ Microbiol 76, 6186–90 (2010).
Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019