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Highlights

- Testing for the first time the nine antimicrobials proposed by EUCAST for *Legionella pneumophila* using the microbroth dilution method with the largest number of *Legionella pneumophila* human isolates.
- Alert on the presence in Italy, as has been found in other countries, of strains of *Legionella pneumophila* with reduced sensitivity to azithromycin.
- Importance of testing for the presence of *Lpe*AB genes associated with reduced sensitivity to azithromycin of *Legionella pneumophila*.

Antimicrobial susceptibility and epidemiological types of *Legionella pneumophila* human isolates from Italy (1987-2020)

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Abstract

Background: Although antimicrobial resistance has not yet emerged as an overarching problem for *Legionella pneumophila* (Lp) infection, the description of clinical and environmental strains resistant to fluoroquinolones and macrolides is a cause of concern. This study aimed to investigate the antimicrobial susceptibility of Lp human isolates in Italy. *Methods:* A total of 204 Lp clinical isolates were tested for sensitivity to nine antibiotics using the broth microdilution assay (BMD). All isolates were typed by sequence-based typing, and *Legionella pneumophila* serogroup 1 (Lp1) isolates by monoclonal antibody subgrouping. Minimum Inhibitory Concentrations data were correlated with the possible source of infection and geographical distribution. The presence of the *lpeAB* efflux pump genes was also investigated. The genome sequences of a subpopulation of isolates showing reduced susceptibility to azithromycin were also analyzed. *Results*: The Lp isolates did not show significant resistance to the tested antibiotics, although a trend towards reduced sensitivity to azithromycin was observed in a subpopulation of 46 strains, most of which belonged to Sequence Type 1 (ST1), the second most widespread ST in Italy. An amplicon of the expected size overlapping the *lpeAB* genes was obtained only in the 46-subpopulation above mentioned. In four of the 46 isolates, sequencing analysis showed the occurrence of amino-acid substitutions already described in other strains. No further mutation was found. *Conclusions:* The presence of Lp strains with reduced susceptibility or resistance to azithromycin should be monitored to predict future trends and suggest to physicians a combined therapy with fluoroquinolones when a poor response to azithromycin is observed.

1.Introduction

Legionella pneumophila (Lp) is responsible for severe pneumonia, called Legionnaires' disease, acquired by exposure to man-made devices producing contaminated aerosol such as showers, spa pools, cooling towers, dental unit waterlines, etc. [1]

An increasing number of cases, clusters, and outbreaks has been notified in several countries, including Italy [2-3]. The infection can be acquired in the community, nosocomial, and occupational settings, with severe sequels or fatal outcomes, particularly among immunocompromised patients [4- 6]. Although provisional scores for clinical and laboratory criteria have been developed, the clinical and radiological picture of Legionnaires' disease (LD) is characterized by interstitial pneumonia that cannot be distinguished from pneumonia caused by other respiratory pathogens [7]. Generally, clinical diagnostic criteria can predict the severity of infection and guide antibiotic therapy. Different and non-specific symptoms of the disease may occur, most often in immunocompromised patients, leading to an incorrect diagnosis and inappropriate antibiotic treatment [8-10]. *Legionella* infects and multiplies within alveolar macrophages, and effective antibiotics must reach the infected cells and achieve inhibitory concentrations therein. Culture-based isolation of *Legionella* from biological samples is rather infrequent due to the poor sensitivity, scarcity of respiratory samples, and time consumption. Failure in *Legionella* isolation can also be due to the collection of respiratory samples after the start of empiric antibiotic therapy, frequently based on fluoroquinolones and macrolides, or a combination of both. All these problems make it difficult to obtain *Legionella* isolates for antimicrobial susceptibility testing. Antimicrobial resistance is not yet a critical problem for *Legionella*, although the recent description of clinical and environmental strains resistant to fluoroquinolones and macrolides has raised attention to possible resistance phenomena. A Lp isolate resistant to fluoroquinolone, associated with antibiotic therapy failure and poor prognosis, was initially reported by Bruin *et al.,* [11]. Both environmental and clinical Lp isolates endowed with decreased susceptibility to azithromycin linked to the presence of the efflux pump *lpeAB* have been documented [12-19]. These and other studies highlighted the importance to investigate the incidence of antibiotic resistance in *Legionella*, to guide the most appropriate antibiotic therapy. In this study, the broth microdilution (BMD) assay was employed to provide an updated survey of antimicrobial susceptibility levels in 204 Lp human strains isolated in Italy. The antimicrobial susceptibility patterns were correlated with typing results, source of exposure, and geographic origin of isolates.

2.Materials and Methods

2.1 Bacterial strains

From 1987 to 2020, 204 human isolates of Lp were collected and stored at -80±5°C at the Italian reference laboratory for *Legionella*, originating from the North (n=129) and from both the Center and the South (n=76) of Italy with different proportions depending on the use diagnosis by culture of the hospital laboratories, but also depending on the different incidence of the disease in our country. Among the 204 *L. pneumophila* strains, 184 were serogroup 1 (Lp1), five serogroup 6 (Lp6), four serogroup 2 (Lp2), three each for serogroups 3 (Lp3) and 10 (Lp10), and there was only one strain for serogroups 5, 7, 8, 9 and 14 (Lp5, Lp7, Lp8, Lp9 and Lp14, respectively). The isolates were classified according to the possible source of exposure, *i.e.* community, nosocomial, and travel-associated. In addition, two reference Lp serogroup 1 strains, namely ATCC 33152 and NCTC 12821, were used as internal controls in antibiotic sensitivity assay, in accordance with the Guidance document on Antimicrobial Susceptibility Testing of *Legionella pneumophila* of the European Committee for antibiotic susceptibility testing [20].

2.3 Monoclonal antibody and sequence-based typing

The serogroup determination of the 204 Lp isolates was obtained using monoclonal antibodies provided by the Carl Gustav Carus University of Dresden, which also developed the Dresden monoclonal antibody (MAb) typing scheme [21-22]

This panel of MAbs was created based on differences in the epitopes of Lp1lipopolysaccharide (LPS), discriminating the following subgroups: Knoxville, Philadelphia, Benidorm, France/Allentown, Olda, Oxford, and Bellingham, Heysham, Camperdown. Monoclonal subgrouping and genomic typing results should match for epidemiologically related strains [23-24]

The sequence type (ST) was determined for both Lp1 and non-serogroup 1 Lp strains by sequence-based typing (SBT) [25-26].

2.4 Antibiotics

The susceptibility of Lp isolates to azithromycin, clarithromycin, erythromycin, levofloxacin, moxifloxacin, ciprofloxacin, rifampicin, doxycycline, and tigecycline (Sigma-Aldrich, town, country) was tested according to the guidance *Legionella* Antimicrobial Susceptibility Testing

(EUCAST). The antibiotics were dissolved as recommended by the Clinical and Laboratory Standards Institute directives for antibiotic susceptibility testing and stored at -30 ±5°C until use [27].

2.5 Antimicrobial susceptibility test

Lp human isolates and the control reference strains were thawed, plated on Buffered Charcoal Yeast Extract (BCYE, Thermo Fisher Diagnostics Limited– Altrincham, United Kingdom) agar plates, and incubated for 48 h at 37±0.5 °C. Bacterial colonies were suspended in buffered yeast extract (BYE, Thermo Fisher Diagnostics Limited – Altrincham, United Kingdom) broth containing the *Legionella* growth supplement (Thermo Fisher Diagnostics Limited – Altrincham, United Kingdom) at an optical density of approximately 0.6 at 600 nm, corresponding to about $5x10^8$ CFU/mL. The bacterial suspensions were appropriately diluted in BYE, and dispensed into 96-well microplates (Falcon, USA). Each well contained a volume of 200 µL, consisting of 100 µL of bacterial suspension at the final concentration of about $5x10^5$ CFU/well, and 100 µL of two-fold serial dilutions of each antimicrobial agent dissolved in BYE broth. Antimicrobials were tested at the following final concentrations (mg/L): azithromycin, 0.015-8; clarithromycin, ciprofloxacin, levofloxacin, and moxifloxacin, 0.0009-0.5; erythromycin and doxycycline, 0.03-16; rifampicin, 0.00005-0.03. Wells containing 200 µL of uninoculated BYE broth or containing bacterial suspension without any antibiotic were used as sterility and growth controls, respectively. The microplates were incubated at 37±0.5°C without agitation, and the optical density at 570 nm was determined after 48 h by spectrophotometer (Multiskan Go, Thermo-Scientific). The minimum inhibitory concentration (MIC) values were defined as the lowest antimicrobial concentration showing 100% growth inhibition compared with the antibiotic-free *L. pneumophila* growth control, as measured spectrophotometrically or visually. The bacterial inoculum was quantified by CFU counts on BCYE agar plates upon serial dilution in sterile distilled water. The inferred epidemiological cut-off values (ECOFF) defined were provided although sufficient data to establish ECOFFs are not currently available for *Legionella* [20].

2.6 Screening assays of *lpeAB* gene

PCR amplification was used to screen for the presence of the *lpeAB* operon, known to encode an efflux pump involved in a macrolide-specific reduced susceptibility [12], using total DNA extracted from *Legionella* isolates. The amplicons were generated using a previously described primer pair

[14] and verified by electrophoresis on 1% agarose gel in tris-borate-EDTA buffer. The *lpeAB* amplicon size (359 bp, overlapping the *lpeA* and *lpeB* genes) was determined by direct comparison with the O'Generuler 1kb (Thermo Scientific, United Kingdom) molecular size ladder. Amplicon identity was determined by DNA sequencing, as outlined hereafter.

2.7 Whole genome sequencing

To identify mutations in target genes commonly involved in antibiotic resistance such as *rpoB, gyrA, gyrB, parC, rplD, rplV,* and the 23S rRNA gene, a subpopulation of 46 strains, consisting of 44 Lp1 and two non-sg1 Lp (Lp5 and Lp7), was analyzed by whole genome sequencing (WGS). Sequencing libraries were prepared using the NextEra XT library prep kit (Illumina) and a 150-bp paired-end sequencing run was performed on the NextSeq-500 (Illumina). Reads were trimmed to keep high-quality bases (Q score >20) using the Sickle software (V.1.33; available at https://github.com/najoshi/sickle). *De novo* assembly was carried out with the SPAdes v.3.15.0 software. Sequences were submitted to the NCBI Genbank (Project ID: PRJNA977629). Genome sequences were then checked for mutations in the targeted genes using an in-house pipeline, as described by Ginevra *et al.* [28] Briefly, reads were mapped on reference sequences using minimap2 (version 2.22) and variants linked to antibiotic resistance were called using freebayes (version 1.3.5). The entire *lpeAB* operon, including the promoter region, was checked in all the 46 isolates, in looking for single mutations. To this aim, genome annotations providing the *lpeAB* positions in the contigs were obtained by PROKKA (version 1.14.5), [29] and the MAFFTs alignment was visualized by Jalview (Version 2.11.3) [30].

3. Results

3.1 Epidemiological types and sources of *Legionella pneumophila* isolates

SBT differentiated the whole collection of 204 Lp isolates in 69 STs, among which the most frequent were ST1 (n=41), ST23 (n=37), ST42 (n=16), ST146 (n=8), ST72 (n=7) and ST435 (n=7). MAb subgrouping of 184 Lp1 isolates highlighted the prevalence of the subgroup Philadelphia (n=97) followed by Benidorm (n=26), Knoxville (n=25), Olda (n=14), France-Alletown (n=13), Oxford (n=5), Bellingham (n=3), and Heysam (n=1) subgroups. Sixty-nine percent of isolates were from community-acquired cases, 24% from nosocomial cases, and 7% from travel-associated cases (Figure 1).

3.2 Antimicrobial susceptibility

The bacterial inoculum was 6.1(±4.1)×10^5 CFU/mL as assessed by plate counts of 15 randomly selected test strains. The MICs of nine antimicrobials were determined for the whole collection of 204 clinical Lp isolates, and the results are reported in Figure 2, where cumulative histograms provide MIC data for Lp1 (n=184) and Lp2-15 (n=20) isolates. MIC ranges, MIC_{50} and MIC_{90} as well as the ECOFF values are shown in Table 1. Notably, all 204 isolates were sensitive to the nine antimicrobials tested, showing susceptibility ranges close to those of the two reference strains. MIC values for Lp1 and Lp2-15 were quite similar for all antibiotics tested. Rifampicin was the most active antimicrobial agent against Lp, with the lowest MIC_{50} and MIC_{90} values of 0.00045 and 0.0018 mg/L respectively. Most of the strains had the same MIC_{50} and MIC_{90} values for fluoroquinolones, equal to 0.03 mg/L. In the macrolide group, erythromycin and azithromycin showed the widest MIC range, and a subpopulation of 46 isolates (44 Lp1, one Lp5, and one Lp7) showed reduced susceptibility to azithromycin with MIC values ranging from 0.25 to 1 mg/L. Tigecycline had the lowest antimicrobial activity against Lp with the lowest MIC values detected for most isolates ranging between 16-32 mg/L. Doxycycline, as tigecycline, was the second antibiotic with the highest MIC_{50} and MIC_{90} values, 4 and 8 mg/L, respectively.

3.3 Detection of *lpeAB* genes and whole genome analysis of *Legionella pneumophila* isolates showing reduced azithromycin susceptibility

PCR amplification of the *lpeAB* efflux pump genes gave an amplicon of the expected size (359 bp) in 46 out of 204 Lp strains tested, all showing reduced susceptibility to azithromycin (MIC range 0.25-1 mg/L; Figure 2). Forty-four isolates were Lp1, 30 of which were Philadelphia, 6 Olda, 6 Oxford, 1 Benidorm and 1 Knoxville, and two were non-Lp1 (one Lp5 and one Lp7). The ST distribution in this subpopulation highlighted that most of the isolates were ST1 (n=34, 74%) followed by ST72 (n=6, 13%), ST701 (n=2, 4,34%), and one for each of the following STs, ST476, ST781, ST1520, ST1904. The minimum spanning trees of the allelic profiles of the STs obtained for the Lp dataset highlighted a correlation between azithromycin MICs and STs (Figure 3A and 3B), showing that the strains with the highest azithromycin MICs belonged either to ST1 or to STs differing for one/two alleles, such as ST72, ST476 and ST781 (Figure 3C). The other genomes of the 46-subpopulation belong to ST701, ST1520, ST1904, ST2212.

3.4 Whole Genome Sequencing

Whole genome sequences were obtained for the sub-population of 46 isolates endowed with reduced azithromycin susceptibility. After de novo assembly, sequences were checked for mutations implicated in antimicrobial resistance. No mutation was found in *rpoB* associated with rifamycin resistance*; gyrA* and *gyrB* encoding DNA gyrase subunits common mechanism conveying fluoroquinolones resistance; *par*C to detect point mutations preventing fluoroquinolone antibiotics from inhibiting DNA synthesis*; rplD, rplV*, and the 23S rRNA responsible for macrolide resistance. In the 46 sub-population, LpeA and LpeB showed the same aminoacidic sequence as LpeA and LpeB of the Paris strain, except for four isolates: 486C (Lp1, ST701), 536C (Lp1, ST701), 2465 (Lp5, ST1520) and 222C (Lp7, ST1904), characterized by MIC values of 1 (both the Lp1) and 0.5 (both the non-sg1). Both LpeA and LpeB in these four strains showed the same aminoacidic substitutions, which have been already described in other strains belonging to serogroup 4 ST1973 (18). A list of these aminoacidic substitutions is reported in Table 2.

4. Discussion

This study describes for the first time the results of antimicrobial susceptibility testing of 204 clinical isolates of Lp, isolated in Italy over 33 years. In Italy, reporting LD has been mandatory since 1983, and although the number of reported cases is continuously increased over the years, LD incidence is not uniform across the country, with 78.7, 55.6 and 14.4 cases per million inhabitants in the northern, central, and southern regions, respectively. This is one of the reasons why in this study the isolates tested for antimicrobial sensitivity are not representative of the whole country, with isolates from the central-northern regions (95,17%) prevailing over those of the southern regions and the two major islands [2]. The isolates collected from community and nosocomial acquired LD cases were more represented than those from travel-associated cases. The community acquired LD cases account for the largest percentage of notified cases in Italy, followed by the travel and nosocomial-acquired cases [2].The BMD assay is one of the methods proposed by the EUCAST Steering Committee and the American Centre for Diseases Control, and it is considered the gold standard for Lp antimicrobial susceptibility testing [31]. This is due to the possible interference in the antimicrobial activity of charcoal, a component added to the BCYE solid growth medium for *Legionella*, which is used for inactivating toxic lipids and peroxides [32]. Recently, Portal *et al.* have developed a promising solid medium without charcoal named LASARUS that

showed a good agreement with BMD [33]. Since a standard protocol for testing antimicrobial sensitivity against Lp has not yet been developed, in this study the BMD method was chosen to investigate the antimicrobial susceptibility of the 204 clinical Lp against the nine antibiotics, as suggested by the EUCAST guidance document [20]. In our experimental setting, the BMD method showed excellent reproducibility and, overall, our data confirm the susceptibility of Lp to the antibiotics usually used in therapy such as fluoroquinolones and macrolides. The results are generally in good agreement with those published in the EUCAST guidance and other authors, referring to the Lp antimicrobial susceptibility test using BMD method both for clinical and environmental isolates, considering that the differences could also be attributed to slight variations in the methodology for testing isolates [4,17,18,19, 34-35]. In our study, isolates belonging to Lp sg1 prevailed and only 20 belonged to Lp 2-15, therefore it was not possible to adequately evaluate a significant difference in MIC values between the two groups with the exception of erythromycin which showed the MIC_{90} two dilution higher for Lp1 (0.5 mg/L) than for Lp2-15 (0.125 mg/L). MIC_{50} for clarithromycin showed only one dilution of difference between Lp1 (0,06 mg/L) and Lp2-15 (0.03 mg/L). Only one dilution of difference was also observed for azithromycin MIC_{90} between Lp1 and Lp2-15, being 0.5 mg/L and 0.25 mg/L, respectively. Similarly, the MIC_{90} of tigecycline had only one dilution of difference between the Lp1 (32 mg/L) and Lp2-15 (16 mg/L). These data agree with other studies and show that Lp1 may have developed strategies that make these strains less susceptible to the action of antibiotics, also explaining their high prevalence in LD cases [14,17-18, 19,34-35]. As reported by other authors [14,18-19], rifampicin was the most active antimicrobial agent against Lp, even using the gradient test [36]. The data obtained from fluoroquinolones are quite uniform with similar MIC values for the three antibiotics belonging to this class in all isolates tested, in good agreement with those obtained from other authors with one dilution of difference in a few cases, and rarely with two dilutions of difference [14,17-19, 34]. In contrast, tigecycline (not tested by BMD method in EUCAST guidance document), a third generation glycylcycline, derived from tetracycline, showed reduced antimicrobial activity against Lp with the highest MIC values detected for most isolates ranging between 16-32 mg/L. Despite the low MICs, tigecycline has been demonstrated useful in therapy in a patient with allergies to both fluoroquinolones and macrolides, indicating that it may be administered as a safe and effective alternative therapy for treatment of LD as well as for improving the patient's condition undergoing to therapeutic treatment with moxifloxacin in combination with azithromycin [37-38]. This could be due to the greater affinity of this antimicrobial for ribosomes compared to other tetracyclines, reaching a significantly higher intracellular concentration than doxycycline, in guinea pig models [39-40]. Doxycycline, derived from the same antibiotic family as tigecycline, was the second

antibiotic with the highest MIC_{50} and MIC_{90} values, 4 and 8 mg/L, respectively. Other authors also found these high values for doxycycline, showing variable results for MIC_{50} and MIC_{90} ranging from 1 to 16 mg/L and 2 to 32 mg/L, respectively [14,17,19].

Also, in our study, as previously reported, the macrolide group was characterized by a broader MIC distribution, with azithromycin showing the broadest [14]. Among macrolides, erythromycin showed the lowest efficacy against our strains, while clarithromycin showed a higher efficacy than azithromycin, consistent with most of the data collected in the literature[14,18-19].

As for the tentative wild-type MIC distribution values, our isolates, both for Lp1 and Lp2-15, were concordant with those suggested by EUCAST for azithromycin, clarithromycin, erythromycin, moxifloxacin, rifampicin and levofloxacin; only ciprofloxacin had 5 Lp1 isolates showing MIC values of 0.06, a higher dilution than that reported by EUCAST [20] For levofloxacin, only the Lp14 isolate showed a quite different value (MIC 0.5 mg/mL) from the 0.125 MIC mg/L value for Lp2-15 group reported by EUCAST guidance [20]. Similarly, one Lp1 isolate had a MIC of 0.125 mg/L for moxifloxacin (one dilution higher than EUCAST guidance). Doxycycline was the only antibiotic with 80% of isolates showing MIC values different from EUCAST tentative wild-type MIC distribution values. These data confirm those obtained by other authors, and we agree with Cocuzza *et al.* to review the tentative of wild-type MIC distribution value for doxycycline [18-19]

Concerning the azithromycin, a subpopulation of 46 strains, characterized by the presence of the efflux pump encoded by *lpeAB* operon, showed a reduced susceptibility, with 5 strains having MIC at 1 mg/L, 37 strains with MIC at 0.5 mg/L and 4 strains with MIC at 0.25 mg/L [14,18-19]. Most of these strains were Lp1 ST1 or STs differing for one allele from ST1, but there were also Lp non-serogroup 1 [14]. In contrast, in the remaining 158 strains with azithromycin MIC values in the expected range (0.03-0.125 mg/L), the *lpeAB* genes were absent, although other authors observed that not all isolates with decreased azithromycin MIC values harbored the *lpeAB* genes [19]. Grouping ST1 and single-locus ST1 variants, such as ST72 (n=6) and ST476 (n=1), all accounted for 87% of *lpeAB*-positive isolates.

Multiple alignment of the LpeA and B proteins highlighted the presence of amino acids substitutions in four out of 46 strains, as those already described in Lp serogroup 4 strains by other authors [15]. The occurrence of these substitutions in isolates other than Lp1 ST1 suggests that they may cause decreased susceptibility to azithromycin also in isolates belonging to other serogroups and STs.

Among the STs with reduced susceptibility to azithromycin, the ST701 was found in two strains (MIC=1mg/L) and has already been described in strains with higher MIC values [15]. Based on what has been so far reported in literature, three further STs can be added to the list of those found in azithromycin less susceptible strains (ST781, ST1520, ST1904) and one more serogroup, the Lp7 [13,15] Additionally, in our study not all the ST1 showed reduced susceptibility to azithromycin, suggesting that this is not a prerogative of ST1 nor Lp1. Apart from the presence of *lpeAB* gene, no other changes were detected in the most relevant genes involved in resistance mechanisms, revealing no resistance to other antibiotics, according to the determined MIC ranges.

Regarding the MAb subgroup, 72% of the Lp1 *lpeAB*-positive strains belonged to the most virulent MAb 3/1 subgroup, with a large prevalence of Philadelphia strains. Strains from nosocomial cases were the most frequent among the 46 sub-population of *lpeAB*-positive strains, accounting for twice as many community-acquired cases.

In conclusion, our data confirm the results already obtained from other studies which demonstrate that the phenomenon of antimicrobial resistance, fortunately, is not yet an emergency even for Italian Lp clinical isolates. However, based on these data, physicians are strongly advised to pay attention to LD cases in which patients do not show rapid improvement in the clinical picture despite administration of azithromycin. It is important to emphasize that the reduced efficacy of azithromycin against Lp seems limited to a small but potentially growing group of STs and a predominant monoclonal subgroup.

It would be important to verify as early as possible the presence of the *lpeAB* efflux pump as well as to quickly verify whether the Lp strain responsible for the infection belongs to ST1 using a real-time PCR assay that specifically identifies the ST1 [39]. This recommendation should be extended also to the environmental *Legionella* monitoring practice. The recent European directive on drinking water 2020/2184 has included the *Legionella* as new parameter to be controlled in the water systems of priority buildings [42]. When applying the water safety plan approach, recommended by the new directive, the alert on the presence of certain STs or monoclonal subgroups should also be taken into account. In recent times, the application of WGS techniques allows the different virulence traits of bacterial isolates present in a water system to be easily and quickly detected. It would therefore be desirable that in the fairly near future risk assessors would use these new techniques, aimed at more rigorous actions when certain isolates are detected in artificial environments, especially when there are people at greater risk of contracting LD.

Furthermore, as preventive action, monitoring the emergence of resistance in *Legionella* is also important due to the threat of environmental spread of antimicrobial resistance caused by the indiscriminate use of antibiotics that pose at serious risk the human health. Some water pathogens

such as *Vibrio, Campylobacter, Salmonella, Shigella, Escherichia coli*, and other opportunistic bacteria, have already been described as integrons carriers [42]. Integrons are genetic elements involved in the spread of antibiotic resistance genes amongst bacterial species, and *Legionella* could also be involved in this genetic transfer. Additionally, considering that *Legionella* lives primarily in the biofilms, several studies have demonstrated that biofilms may represent an ideal substrate for the dissemination of biocide resistance cassettes within the bacterial population [43].

Finally, it has been shown that there could be a possible relationship between biocide exposure and antibiotic resistance selection [43-45] and, since *Legionella* is constantly exposed to disinfectants in engineered water systems, the occurrence of these events must be considered and carefully monitored in the future.

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Figure legends

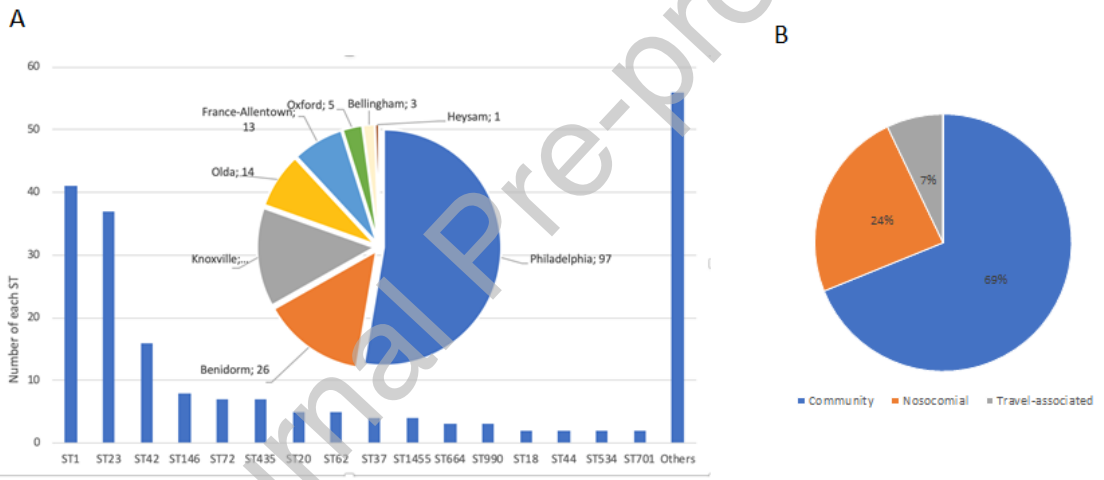

Figure 1. (A) The histogram represents the number of each STs, determined in the collection of 204 *L. pneumophila* isolates. The pie chart represents the distribution of *L. pneumophila* serogroup 1 monoclonal subgroups. (B) The pie chart represents the settings of infection of the cases from which the strains were isolated.

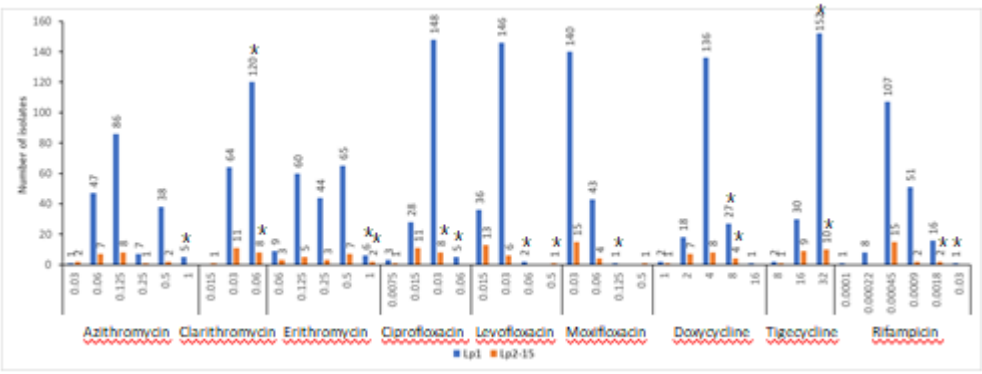

Figure 2. Distribution of minimum inhibitory concentrations (MICs) of the nine antibiotics, used in this study for *L. pneumophila* serogroup 1 and *L. pneumophila* 2-15 clinical strains. Asterisks indicate the MICs corresponding to the deduced epidemiological cut-off values (ECOFF).

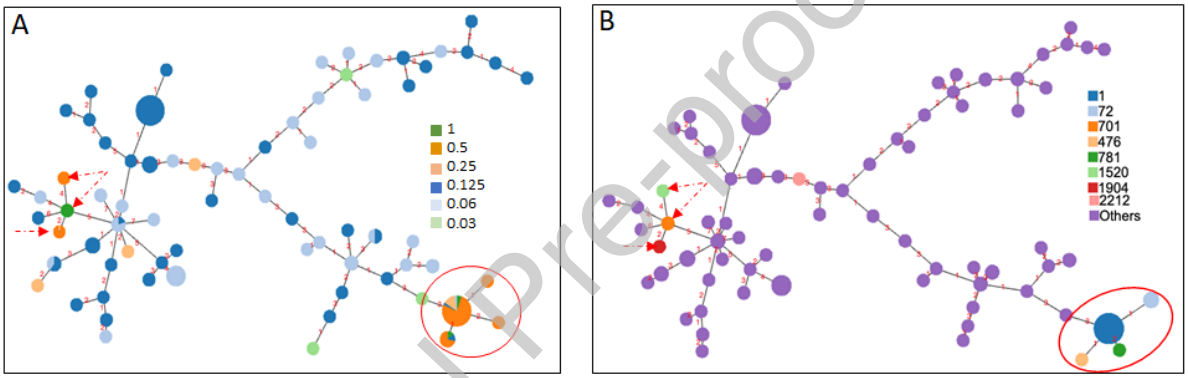

Figure 3. Minimum spanning tree (MST) of *L. pneumophila* clinical isolates (n=204) based on the allelic differences among the STs. The size of the nodes is proportional to the number of strains included in each node. On the branches, the difference in the number of different alleles is reported. (A) MICs distribution is represented by the different colours of the nodes. The highest MICs are highlighted by the red circle and arrows. (B) STs linked to the highest MICs are indicated by the red circle and arrows.

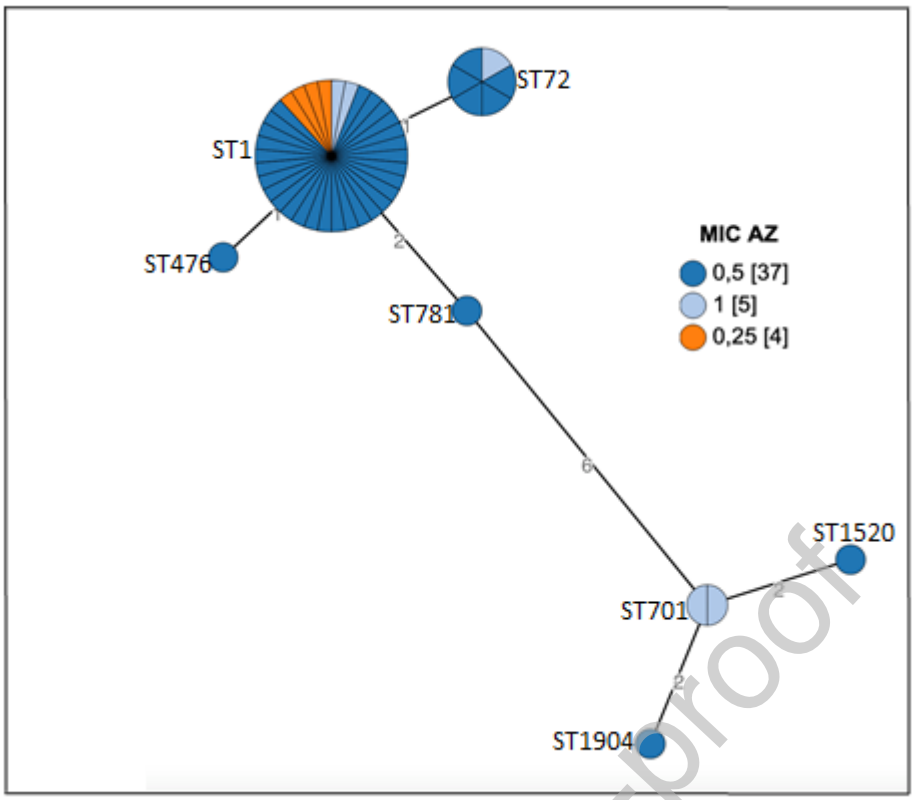

Figure 4. Minimum spanning tree (MST) of the 46 *Legionella pneumophila* strains showing reduced susceptibility to azithromycin. Colours of the nodes indicate the MIC value as in the legend, and close to the nodes the STs are indicated. On the branches, the number of allelic differences is reported.

Table 1. MIC ranges, MIC_{50}, MIC_{90} and ECOFF values determined for all the Lp sg 1 and all the Lp sg 2-15 for each antibiotic. MIC ranges of reference strains are also reported

Antimicrobial agent	Lp sg 1 MIC range (mg/L)	Lp sg 1 MIC50 (mg/L)	Lp sg 1 MIC90 (mg/L)	Lp sg 1 ECOFF (mg/L)	Lp sg 2-15 MIC range (mg/L)	Lp sg 2-15 MIC50 (mg/L)	Lp sg 2-15 MIC90 (mg/L)	Lp sg 2-15 ECOFF (mg/L)	Lp ATCC 33152 MIC range (mg/L)	Lp NCTC 12821 MIC range (mg/L)
Azithromycin	0.03-1.00	0.125	0.5	1	0.03-0.5	0.125	0.25	0.5	0.06-0.25	0.06
Clarithromycin	0.015-0.06	0.06	0.06	0.06	0.03-0.06	0.03	0.06	0.06	0.03-0.06	0.03-0.5
Erythromycin	0.06-1	0.25	0.5	1	0.06-1	0.25	0.125	1	0.125	0.125
Ciprofloxacin	0.0075-0.06	0.03	0.03	0.06	0.015-0.06	0.015	0.03	0.03	0.015-0.03	0.015-0.03
Levofloxacin	0.015-0.06	0.03	0.03	0.06	0.0075-0.06	0.015	0.03	0.06	0.015-0.03	0.015-0.03
Moxifloxacin	0.03-0.125	0.03	0.06	0.125	0.03-0.6	0.03	0.06	0.06	0.03-0.06	0.03
Doxycycline	1-8	4	8	8	1-8	4	8	8	4-8	2-8
Tigecycline	8-32	32	32	32	8-32	16	32	32	16-32	8-32
Rifampicin	0.00022-0.0018	0.00045	0.0018	0.0018	0.00045-0.0035	0.00045	0.0018	0.0035	0.00057-0.015	0.00057-0.015

The MIC range values for LP sg 1 and LP sg 2-15 are highlighetd with colours for easier comparison.

Table 2. Amino acid substitutions in LpeA and LpeB of *L. pneumophila* strains compared to *L. pneumophila* Paris reference strain.

Serogroup/subgroup	*Number of strains (N tot = 46)*	AZ [8 - 0.015]	ST	lpp2879(*lpeA*)	lpp2880(*lpeB*)
Lp1 Benidorm	1	1	701	G9S;V26I;S34G;Y39H;S40L;E45V;S46N;I47V;P56T;S59R;T102A;K145R;S197P;A219T;L230M;D259E;T267A;S268N;L272V;I287L;L298I;A307V;E316Q;T333I;T344A;T352I;S353T;S355G	S33Q;N41H;S50N;I63V;A86T;A89T;T107A;V114I;P121Q;T127V;A244T;K247R;N257S;A259S;I296V;K309N;I327L;I380L;D418E;A488E;K503R;H507Q;N510S;L524I;D580N;A581T;A585S;N586S;H610N;S612T;I616L;V644I;S651T;H662N;T670S;V676I;A684E;A685T;N687S;N714S;I741L;S764P;V765I;H798R;G812T;T813I;T814A;M818I;I827T;K921Q;S933A;G978S;L979V;I1000L;M1002L;T1005K;G1007K;K1008I;K1009N;R1010S;C1011S;S1012K;K1013N;E1014K;-1015D;-1016L;-1017F;-1018K;-1019M;-1020K
Lp1 Philadelphia	1	1	701	G9S;V26I;S34G;Y39H;S40L;E45V;S46N;I47V;P56T;S59R;T102A;K145R;S197P;A219T;L230M;D259E;T267A;S268N;L272V;I287L;L298I;A307V;E316Q;T333I;T344A;T352I;S353T;S355G	S33Q;N41H;S50N;I63V;A86T;A89T;T107A;V114I;P121Q;T127V;A244T;K247R;N257S;A259S;I296V;K309N;I327L;I380L;D418E;A488E;K503R;H507Q;N510S;L524I;D580N;A581T;A585S;N586S;H610N;S612T;I616L;V644I;S651T;H662N;T670S;V676I;A684E;A685T;N687S;N714S;I741L;S764P;V765I;H798R;G812T;T813I;T814A;M818I;I827T;K921Q;S933A;G978S;L979V;I1000L;M1002L;T1005K;G1007K;K1008I;K1009N;R1010S;C1011S;S1012K;K1013N;E1014K;-1015D;-1016L;-1017F;-1018K;-1019M;-1020K

Lp 5	1	0.5	1520	G9S;V26I;S34G;Y39H;S40L;E45V;S46N;I47V;P56T;S59R;T102A;K145R;S197P;A219T;L230M;D259E;T267A;S268N;L272V;I287L;L298I;A307V;E316Q;T333I;T344A;T352I;S353T;S355G	S33Q;N41H;S50N;I63V;A86T;A89T;T107A;V114I;P121Q;T127V;A244T;K247R;N257S;A259S;I296V;K309N;I327L;I380L;D418E;A488E;K503R;H507Q;N510S;L524I;D580N;A581T;A585S;N586S;H610N;S612T;I616L;V644I;S651T;H662N;T670S;V676I;A684E;A685T;N687S;N714S;I741L;S764P;V765I;H798R;G812T;T813I;T814A;M818I;I827T;K921Q;S933A;G978S;L979V;I1000L;M1002L;T1005K;G1007K;K1008I;K1009N;R1010S;C1011S;S1012K;K1013N;E1014K;-1015D;-1016L;-1017F;-1018K;-1019M;-1020K
Lp 7	1	0.5	1904	G9S;V26I;S34G;Y39H;S40L;E45V;S46N;I47V;P56T;S59R;T102A;K145R;S197P;A219T;L230M;D259E;T267A;S268N;L272V;I287L;L298I;A307V;E316Q;T333I;T344A;T352I;S353T;S355G	S33Q;N41H;S50N;I63V;A86T;A89T;T107A;V114I;P121Q;T127V;A244T;K247R;N257S;A259S;I296V;K309N;I327L;I380L;D418E;A488E;K503R;H507Q;N510S;L524I;D580N;A581T;A585S;N586S;H610N;S612T;I616L;V644I;S651T;H662N;T670S;V676I;A684E;A685T;N687S;N714S;I741L;S764P;V765I;H798R;G812T;T813I;T814A;M818I;I827T;K921Q;S933A;G978S;L979V;I1000L;M1002L;T1005K;G1007K;K1008I;K1009N;R1010S;C1011S;S1012K;K1013N;E1014K;-1015D;-1016L;-1017F;-1018K;-1019M;-1020K
Lp 1 Philadelphia	3	1	1	-	-
Lp 1 Knoxville	1	0.5	1	-	-
Lp 1 Olda	8	0.5	1	-	-
Lp 1 Olda	1	0.5	476	-	-
Lp 1 Oxford	1	0.5	781	-	-
Lp 1 Oxford	1	0.5	72	-	-
Lp	1	0.	1	-	-

1 Oxford		5			
Lp 1 Philadelphia	1	1	72	-	-
Lp 1 Philadelphia	16	0.5	1	-	-
Lp 1 Philadelphia	5	0.25	1	-	-
Lp 1 Philadelphia	4	0.5	72	-	-