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Occurrence of macrolides resistance in *Legionella pneumophila* ST188: Results of the Belgian epidemiology and resistome investigation of clinical isolates

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ABSTRACT

Objectives: The incidence of Legionnaires' disease steadily increases worldwide. Although *Legionella pneumophila* is known as pathogenic, systematic investigations into antibiotic resistance are scarce, and reports of resistance in isolates are recently emerging.

Methods: Clinical cases and metadata reported to the Belgian National Reference Centre between 2011 and 2022 were retrospectively analyzed. A total of 283 clinical isolates were typed by core genome multilocus sequence typing (cgMLST). Acquired genes or mutations triggering resistance were extracted from all of them.

Results: The number of Legionnaires' disease cases has increased in Belgium. Urinary antigen testing remains the main used test, but polymerase chain reaction and serology allow the diagnostic in 14.8% and 2.4% of cases, respectively. cgMLST showed a good discrimination between sequence typing (ST) and minimal variation for ST47 isolates, whereas ST1s were more diverse. Genotypic screening identified a 23S ribosomal RNA mutation linked to a high-level macrolide resistance in one isolate of ST188, which is genetically closed to resistant isolates from France.

Conclusion: The increase in incidence is of concern and likely an under-estimate due to the reliance on urine antigen testing. Routine typing by cgMLST allows good discrimination and the first clinical isolate reported as resistant for macrolides was cultured, underscoring the need to define resistance breakpoints and incorporate antimicrobial susceptibility testing as routine clinical investigation practice.

Introduction

Legionella pneumophila (*Lp*) is an ubiquitous environmental bacterium responsible for infections associated with human activities. Inhalation of aerosols from contaminated man-made freshwater sources are the main source of infection. Higher levels of *Lp* are associated with water stagnation and biofilm formation, cooling-towers, air conditioning devices and bathroom showerheads represent the most common sources. It can cause either severe pneumonia (community-acquired or nosocomial) called Legionnaire's disease (LD) or a flu-like syndrome called Pontiac fever, although extra-pulmonary presentations (hepatic or neurological) can be observed. Serogroup 1 (SG1) of *Lp* is responsible for more than 80% of LD, more commonly diagnosed in male patients over 50 or in immunocompromised patients [1].

The major increase in LD worldwide over the last decade is of concern and a focus of surveillance by the European Centre for Disease Prevention and Control [1,2], particularly, because it has been hypothesized that climate change will result in greater en-

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vironmental colonization [2,3]. In Belgium, every case is reported to the public health authority and an epidemiologic investigation is started to find the source of infection and prevent epidemics. The Belgian National Reference Centre (NRC) plays a role in coordinating testing and confirmation of clinical cases and linking patient samples and environmental sources through typing. Genotypic investigation of *Lp* is so far based on sequence-based typing (SBT) and previous outbreak investigations revealed that some sequence types (STs) are found more often in clinical cases [4]. A core genome multi-locus sequence typing (cgMLST) comparing 1521 alleles (compared with seven for SBT) was developed for greater discrimination of isolate clusters and is now widely used in *Legionella pneumophila* epidemiologic investigations [5–8].

Legionella is susceptible to macrolides, rifampicin, and fluoroquinolones, which are used for empirical treatment. To date, fluoroquinolone resistance (mediated by GyrA mutation) has only been reported for one clinical isolate [9]. Although high-level macrolide resistance had previously only been induced *in vitro* [10], it has recently been found in environmental isolates [11], in both cases, primarily mediated by 23s rRNA (*rrl* gene) mutation in all three operons. Resistance toward rifampicin has been described in *Lp* and non-*pneumophila* species [12]; however, rifampicin is relatively toxic and now reserved for combination therapy for severe infections. Nevertheless, the lack of antibiotic susceptibility testing standards for *Lp* hinders systematic resistance determination and follow-up [13].

The cases reported to the NRC between 2011 and 2022 were retrospectively analyzed to review the epidemiologic data of the Belgian NRC for diagnostic and surveillance capacities. The available clinical isolates were sequenced by whole genome sequencing (WGS) for typing by cgMLST and using genotypic approach to assess antimicrobial resistance of *Lp* in Belgium in absence of standardized method to perform antimicrobial susceptibility testing (AST) routinely.

Methods

Epidemiologic data

The Belgian NRC is divided between two centers: the Universitair Ziekenhuis of the Vrij Universiteit Brussel and the associated center Laboratoire des Hôpitaux Universitaires de Bruxelles. Both centers receive samples and isolates from any Belgian laboratories.

Patient available metadata included date of birth, gender, and address, with additional details including symptoms, immune status, nosocomial, community-acquired or travel-associated, previous results for urinary antigen testing (UAT), or polymerase chain reaction (PCR). Inclusion was based on the European definition of LD from 2012 [14]. Incidence was calculated based on data collected by the Belgian Institute of Health, Sciensano. Linear regression and Spearman correlation of incidence were performed in R.

Laboratory methods

WGS was performed on 283 unduplicated clinical isolates, previously archived at −80°C. Strains were grown on buffered charcoal yeast extract medium (Oxoid) at 35°C, with supplemented 5% CO_2 and humidified atmosphere (2-4 days). Confirmation of *Lp* was established by matrix-assisted laser desorption/ionization-time of flight spectra of single colonies (Brücker).

DNA extraction and SBT were performed as previously described [4,6]. WGS library preparation and sequencing were performed by short-read methods using Illumina technologies [6]. Sequence quality was assessed with FastQC software (version 0.11.4). PCR used the KAPA Hyper Plus kit (Kapa Biosystems, Wilmington, MA, USA).

Data analysis

Raw data were uploaded and trimmed on the Bionumerics software (BNs) version 8.1 (Biomérieux, France). *De novo* assemblies were performed using SPAdes [6] or SKESA [15]. Acceptable quality assessment was checked by assembly quality metrics report from BNs and controlled by CheckM online pipeline [16] (Supplementary data 1). Criteria for quality metrics acceptance are reported in the Supplementary data 1. cgMLST analysis was carried out using a gene-by-gene approach of 1521 core genome alleles, as previously described [7]. The alleles called on cgMLST was more than 97% of expected number (Supplementary data 1). Reference genomes were downloaded from Genbank: isolate *Lp* Philadelphia 1 ST36 (Accession number (AN): NC_002942.5); *Lp* Corby ST51 (CP000675.2); and *Lp* Paris ST1 (NC_006368.1). Six French isolates ST188 were downloaded from PRJEB51253. A global phylogenetic analysis was performed for categorical values differences and clustering by UPGMA on the BNs and visualized on iTOL v6 [17]. A minimum spanning tree on categorical values was performed on BNs for 209 isolates, grouping the major clades observed on previous tree added to the eight isolates ST188 (two Belgian and six French). A whole genome single-nucleotide polymorphism (SNP) analysis was performed to compare available ST188 isolates of our collection with the six French isolates (two clinical isolates, LEG1112 and LEG412, added to three environmental isolates related to LEG412).

Acquired genes conferring antibiotic resistance were retrieved by the online CARD resistance plugin [18]. Genes for which non-synonymous mutations were described as mutation-mediated antimicrobial resistance were extracted from *de novo* assemblies using the BNs module. The following encoding genes were investigated: DNA Gyrase subunit A (*gyrA*) and subunit B (*gyrB*), ParC subunit of DNA topoisomerase IV (*parC*), β subunit of bacterial RNA polymerase (*rpoB*), 50S ribosomal subunit L4 (*rplD*), and 50S ribosomal subunit L22 (*rplV*). Each gene was aligned then translated to the reference sequence *Lp* Paris strain; synonymous mutations were not reported.

The three copies of 23S ribosomal RNA (23S rRNA), coded by *rrl* genes, were also analyzed with BNs module. For 115 isolates, the copies of 23S rRNA could not be identified after *de novo* assembly due to inherent challenges of assembling repeated regions in short reads [19]. Therefore, mapping of sequences to reference strain 23S rRNA with a similarity threshold of 75% was used and a copy of the *rrl* gene for each of these isolates was extracted. All fastQ or fasta files were published on NCBI under the Bioprojects PRJNA1132000, PRJEB52784, and PRJNA1073851.

Investigation of macrolide resistance for isolate LEG1112

Minimal inhibitory concentrations (MICs) of erythromycin, clarithromycin, azithromycin, josamycin, levofloxacin, and rifampicin were determined in quadruplicate for isolate LEG1112 by the broth microdilution method currently being proposed as the international standard by the ESCMID Study Group for Legionella Infections (ESGLI) [20]. Oxford nanopore technology long-read sequencing was performed as previously reported [21].

Results

Between 2011 and 2022, 618 cases of Legionellosis were reported by the NRC: only 18 due to non-*pneumophila* species and the remaining 99.7% caused by *Lp* (80.4% of which were SG1) (Figure 1). The gender ratio was 2.66:1 (male:female) and the median age was 61 (11-94) years. Pneumonia or respiratory symptoms were reported for 66.2%. Extra-pulmonary cases were rare; of note were two hepatitis, one neurologic presentation, and a cellulitis.

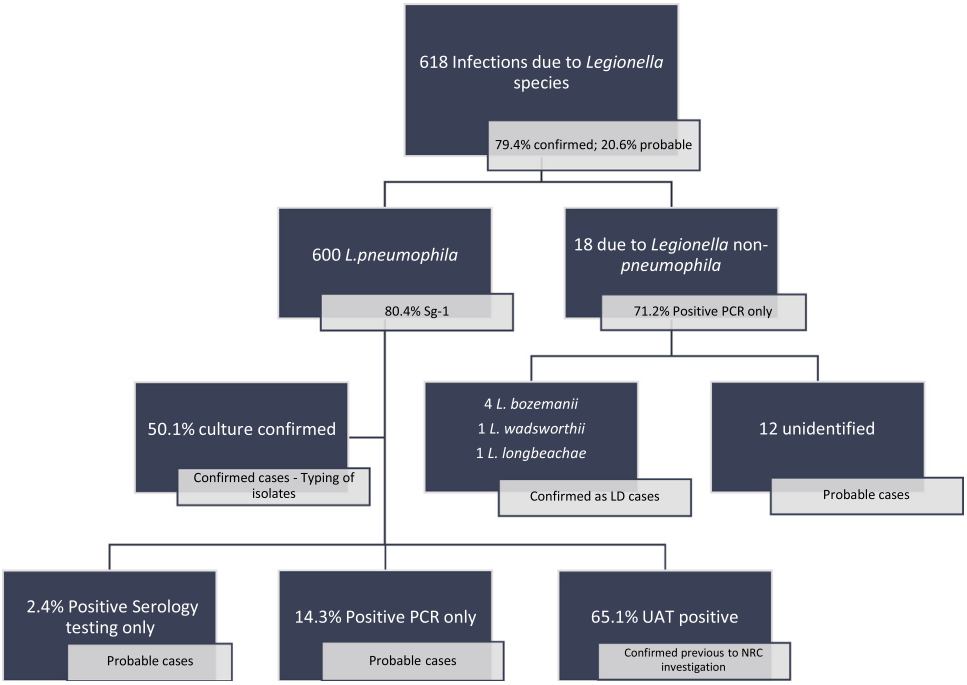

Figure 1. Diagram representing the confirmed and probable cases of LD reported by the Belgian National Reference Centre according to the European Centre for Disease Prevention and Control definition (16); the identified species are specified, as well as the test used for diagnostic.
LD, Legionnaires' disease; PCR, polymerase chain reaction.

The reported mortality was 14%, but this information was provided only in 29% of cases. The majority of the reported LD cases were community-acquired (45.1%; definite or presumed), 6% were nosocomial or health care–associated, and 12.1% were travel-associated (including 1.3% domestic travel); however, no data were available for 33.7%. According to the case definition of European Centre for Disease Prevention and Control, 79.4% cases were confirmed and 20.6% probable [14]. The majority of cases (65.1%) were confirmatory arising from patients who were UAT-positive referred to the NRC for investigation. For *Lp* infections, 33.2% of cases were UAT- and culture-positive. PCR was positive in 79.7% of all cases (the only positive test for 14.8% of the cases), whereas culture was positive in 50.1% (Figure 1). Only 2.4% cases were set as probable by serology testing (all performed during an outbreak).

The incidence of LD increased from 1.62 per 100.000 in 2011 to 3.49 per 100.000 in 2022. A slight decrease was observed during the COVID pandemic (Figure 2). However, a positive linear time trend was detected for the total annual number of cases between 2011 and 2022 (estimated yearly trend = 28, 95% confidence interval [18-38]; Spearman correlation = 89%, $P < 6.10^{-6}$) (Supplementary result 1). The proportion of global cases reported to NRC ranged from 5% to 30%; generally, the trends of NRC followed the national trend [23] (Figure 2). A significant increase of the annual number of LD cases was observed between the beginning of the period (2011) and the end (2022) as 26-94 cases per year, respectively (Figure 2).

SBT was available for 348 samples. The major STs were ST1 and ST47, as previously described in Belgium and Europe [4,22] (Figure 3a), followed by ST42, ST23, and ST921, the last one due to the occurrence of an outbreak. STs that were represented by less than three isolates are grouped into the label ST-Others (ST-O). The geographical distribution of major STs were represented on a map according to the province of origin (Figure 3b). Five outbreaks were described during this period, with a total of 112 reported cases due to specific STs when isolates could be typed (ST48, ST54, ST703, ST921), but one ST remained unknown as causing mainly Pontiac fever, resulting in a lack of respiratory samples to investigate (Figure 3b).

In total, 283 isolates were typed by WGS (Supplementary data 1) and were analyzed by cgMLST (Figure 4a). Excluding the ST-O group, 18 different STs were represented in the phylogenetic analysis. Most STs clustered together despite the higher discrimination of cgMLST. Of note, the ST6 isolates and LEG461 (ST1735) cluster within the ST1 clade and all ST921 isolates were related to the Evergem outbreak, except LEG1086, which was isolated 9 months after the end of this outbreak, at a distance of 10 km (Figure 4a).

To better evaluate the discrimination of isolates within the nine clades, 209 of them were included in a minimum spanning tree for categorical data (Figure 4b). The analyses showed that isolates within a same ST are separated by up to 303 allelic differences (ADs). ST47 isolates that showed a very closed relatedness for most of them formed a large cluster of 41 isolates. ST1 harbors much diversity and two clusters, one gathering seven isolates from a clone that circulated in Brussels Capital and Hainaut between 2016 and 2022, already investigated by a previous study [6]. The other with isolates from mixed origin between 2012 and 2022. This shows an improvement of discrimination between SBT and cgMLST, even for ST1. The isolate LEG1086 remained outside the cluster formed by the ST921 2019 outbreak, with 12 AD differences showing good discrimination by cgMLST. ST23 formed one cluster of six isolates unrelated epidemiologically but show close homology (<39 ADs). ST42 did not form any clusters but isolates had limited AD distances (47 ADs). A cluster of four isolates of ST93 is shown, all from Hainaut in 2021 and 2022, suggesting the circulation of a clone that may represent an unreported outbreak.

A total of 55 isolates, all ST1 and ST6 (n = 5) and ST1735 (n = 1), harbored the efflux pump LpeAB related to diminished macrolide susceptibility (Figure 4a). Interestingly 49 isolates also carried the gene $bla_{oxa\text{-}29}$ (Supplementary Data 1) and eight isolates harbored the gene *aph*(9)-Ia of unknown aminoglycoside specificity, both of which have been described in *Legionella* [24,25].

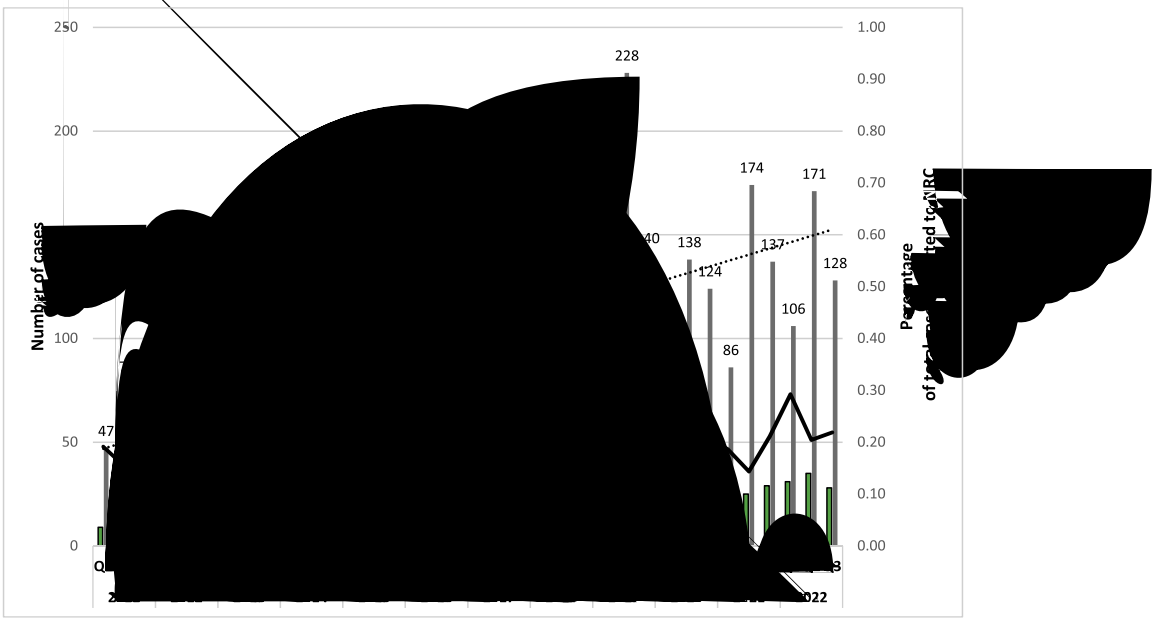

Figure 2. Number of cases of Legionnaires' disease in Belgium between 2011 and 2022. Graphics represent the total number of cases reported by the Belgian Institute of Health, Sciensano (grey bars) [22], and the number of cases reported by the NRC (green bars) throughout time. Time lapses were divided in three periods of 4 months within a year: Q1-Q2-Q3. The proportion of cases reported to the NRC is represented by a full black line. The trend of total number of cases is represented by a dashed black line. NRC, Belgian National Reference Centre.

No previously described fluoroquinolone resistance-mediating mutations in gyrA, gyrB, and parC were found [26,27] (Table 1). A series of isolates (n = 63) harbored a non-resistance polymorphism in GyrA, characterized by an insertion of nine nucleotides forming a repeat sequence c.2542_2550insAGAAGAATT, inserting three amino acids between positions 849 and 867 in the translated sequence (data not shown). The rpoB gene, linked to rifampicin resistance, harbored various polymorphisms but none involving the area that confers resistance in *Legionella* species (between AA 528 and 547) (Table 1) [12]. The L4 (*rplD*) ribosome accessory protein harbored no mutations previously described (Table 1). In 40.7% (n = 115) of the isolates, short-read assembly combined all sequences into a single *rrl* gene copy, leaving 58.6% (n = 166) to be fully investigated. However, the screening of *rrl* sequence for one isolate, LEG1112, showed the presence of the punctual mutation, A2052G, known to confer high-level macrolide resistance [10,11]. Erythromycin, clarithromycin, and azithromycin MICs were confirmed as 1024 mg/l, whereas josamycin MIC was reported at 8, and combined long-read sequencing confirmed afterward that all three copies of *rrl* carried A2052G.

This isolate was further investigated by cgMLST comparing it with six of the macrolide-resistant ST188 environmental isolates found in France in 2021, 2 years after the Belgian case was reported (Figure 4b). To prove the close relatedness between ST188 isolates, a whole genome SNP was performed and although the resistant isolates clustered altogether (less than five SNPs), an additional Belgian ST188 isolate (LEG412, collected in 2012) and its environmental related strains, with no rrl mutations, did not (Supplementary result 2).

Discussion

The Belgian NRC has a major role in the surveillance and investigations of LD cases. Samples and isolates are sent on voluntary basis; therefore, not all LD cases reported to the Belgian Institute of Health can be further investigated. Consequentially, the results presented here are biased at enrollment. However, the general trend of cases is similar between the NRC and the health institute. It is also comparable to the trends of increase of LD cases all over Europe, Australia, and the USA in the last decade and similar incidence associated with seasonality in summer and beginning of autumn [1,2]. UAT remains the main reported diagnostic tool, in accordance with European epidemiology dominated by *Lp* SG1, proving that the increase in incidence cannot be linked only to PCR progression, although it has helped covering the gap in diagnostic for non-SG1 and *Legionella* sp. [1,23]. A decrease in the NRC trend was observed in 2020 and 2021, as in other countries [1], and probably associated with containment measures during the COVID-19 pandemic and lower testing capacity. Thereafter, the incidence seems to increase again proportionally with the initial figures, leading to the hypothesis of geographical factors or climate factors linked to this phenomenon [2,3]. Nevertheless, we did not observe significant difference between Belgium regions because the size of the country, its global climate, and the number of cases are too limited to extrapolate conclusions. However, there is a lack of scientific evidence for the trigger of sudden *Lp* proliferation in plumbing systems and water facilities, leading to ineffective prevention of outbreaks [28], and five outbreaks occurred during this period. The main hypothesis remains to be increased water temperatures in summer associated with increased iron released by pipe corrosion [28].

Gender ratio, median age, and mortality are similar to other studies [1,2]. Respiratory symptoms remain the predominant clinical presentation. During outbreaks, a majority of Pontiac fever cases could be observed, mainly diagnosed thanks to the epidemiologic link to the source of infection identified by public health investigations. This highlights that many cases probably remain undiagnosed because of their benignity and lack of further notice. *Lp* is difficult to culture, and, in this study, only half of the cases were culture-positive and 14.8% of cases were only detected by PCR. Only 2.4% cases were diagnosed by serology, always in the context of an outbreak. Despite its lack of sensitivity, serology remains very helpful when a large number of patients have to be tested and should still be used in such situation. It seems obvious that all tests remain important in the testing arsenal of the NRC.

Most provinces of Belgium report isolates of various STs, with a predominance of ST1 in the Brussels area and ST47 in the

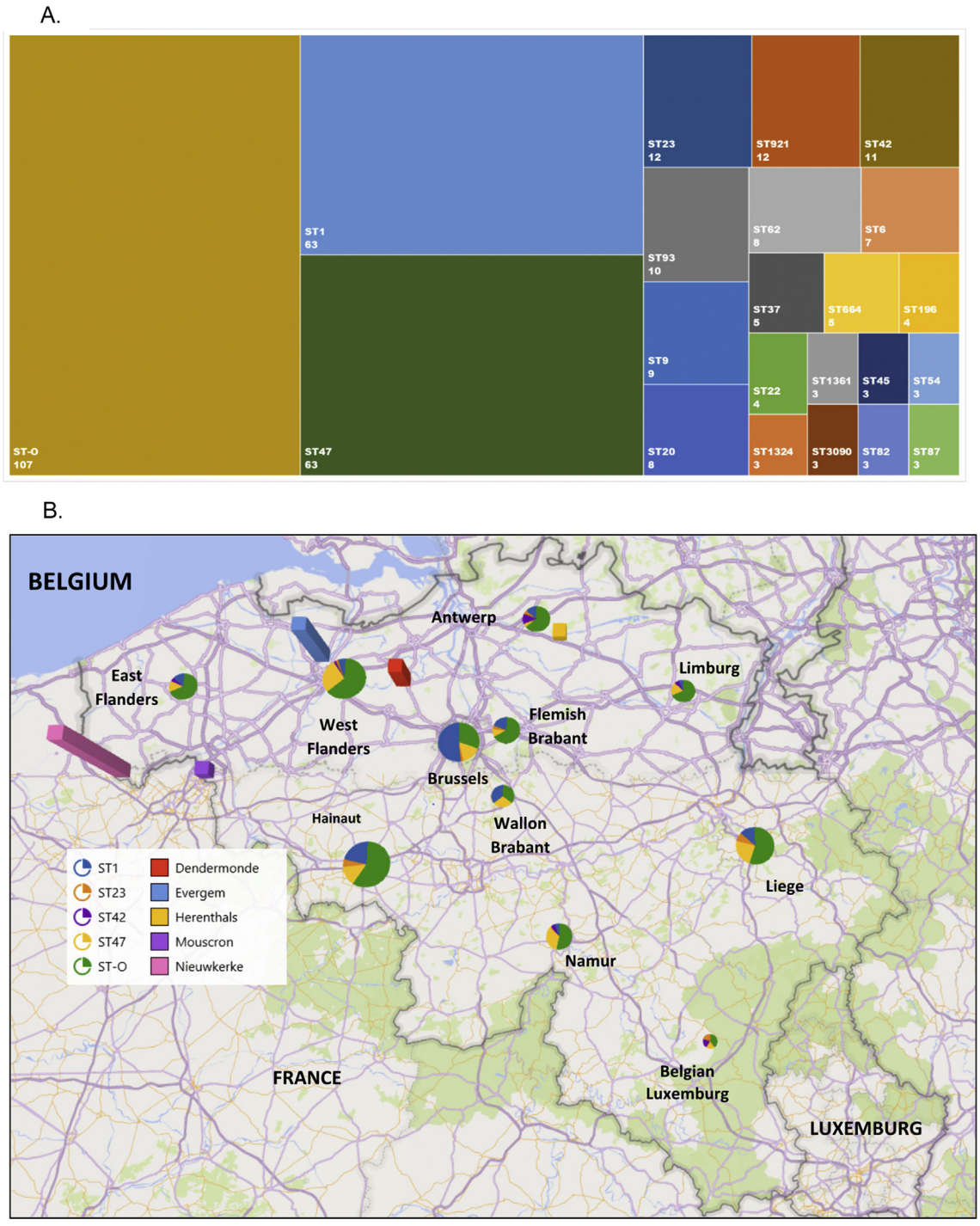

Figure 3. Prevalence of *L. pneumophila* ST and their distribution through Belgian provinces, with graphical representation of outbreaks that occurred between 2011 and 2022. (a) STs that were isolated more than two times (including travel associated) and ST-O. (b) Map representing the distribution of sequence types of *L. pneumophila* through Belgian provinces between 2011 and 2022. ST distribution is represented by a disk filled proportionally to the number of strains isolated in each province for the more frequent STs: ST1, ST47, ST23, ST42, and ST921. STs isolated only once or twice are represented by the group ST-O. Other STs are not represented. Epidemics are represented by a bar graph proportional to the number of cases reported in the city where they occurred.
STs, sequence types; ST-O, ST-Others.

Ghent area; however, generally, no ST is significantly related to a region (Figure 3b). Nevertheless, the ST921 linked to the epidemic of May 2019 was once more reported in early 2020 in the same area but the isolate harbored 12 ADs with the epidemic clone. Because *Legionella* form biofilms in water tanks and pipelines, the persistence of clones in reservoirs is a well-known characteristic of *Lp*. Because it is generally associated with a slow genetic evolution, the appearance of this isolate might only be linked to the divergence of an older common ancestor in the same area.

Until now, SBT is the routine reference method for discriminating isolates but the results are often unconfirmed by WGS and technically limited [6,7]. The link between a patient who tested positive and a source has to be inferred not only by this low discriminatory test but also by a somewhat subjective epidemiologic linking [4,6,10,29]. Deeper genomic epidemiology is needed to bet-

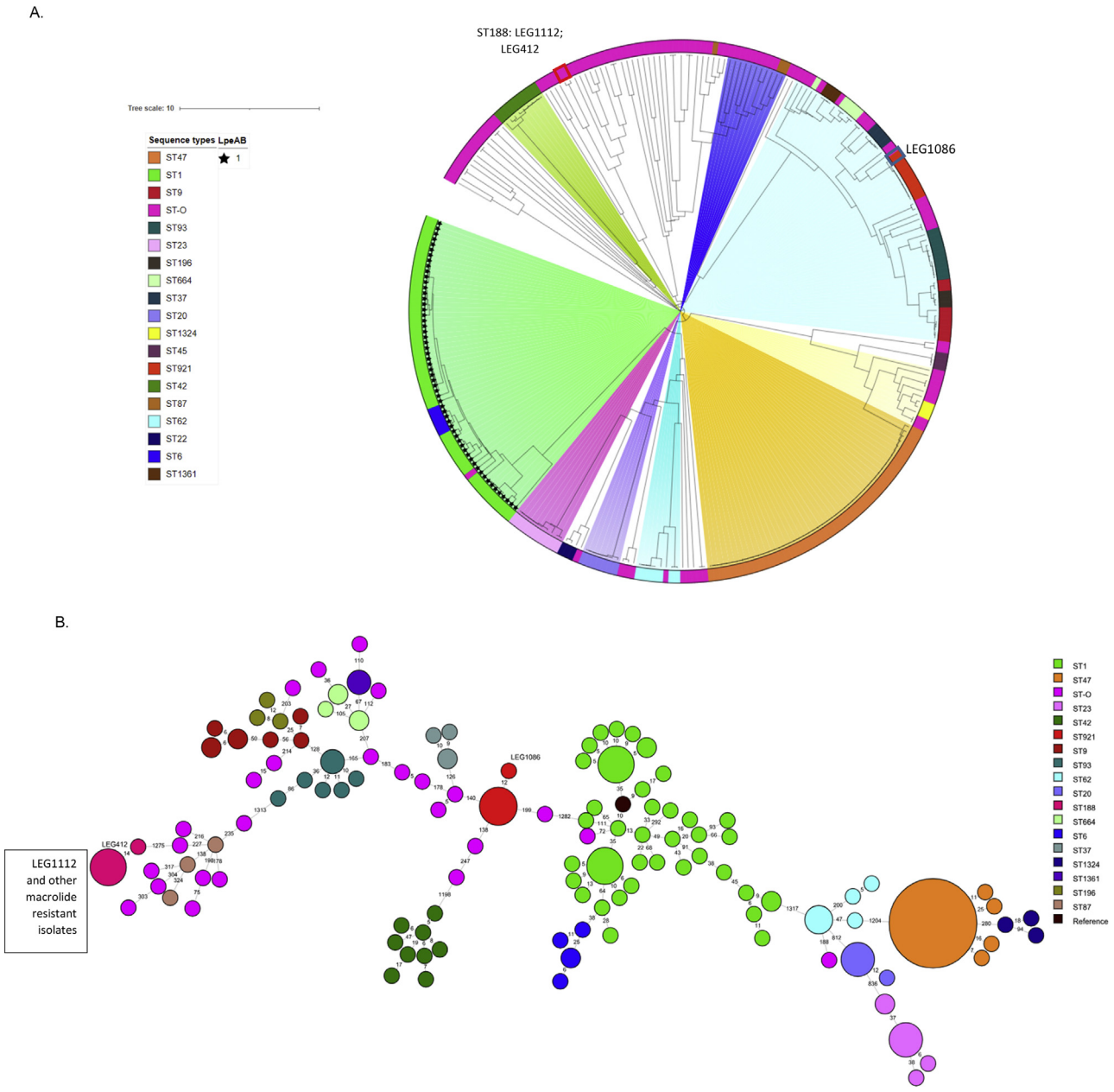

Figure 4. (a) Dendrogram representing the distribution of *Lp* strains isolated between 2011 and 2022 through clinical LD cases in Belgium. Analysis was run on Bionumerics by calculation of categorical values as distance and clustering by UPGMA; branch quality was assessed by cophenetic correlation. The scale represents branch length according to categorical values differences. Visualization was performed on iTOL v6. Internal coloring of branches shows the nine major clades. Extern coloring represents the sequence types determined by sequence-based typing. The three reference isolates are *Lp* Philadelphia, *Lp* Paris, and *Lp* Corby. The presence of the efflux pump LpeAB is represented by a black star added on the top of the branch. (b) Minimum spanning tree for categorical data of 315 isolates corresponding to all isolates included in the clades highlighted by initial analysis added to ST188 environmental strains resistant toward macrolides and from our collection (LEG1112; LEG412) (Figure 4a). Branches were logarithmically scaled and labels correspond to the number of allelic differences between each node. All isolates less than 5 allelic differences from each other are represented in the same node, which size is proportional to the number of isolates included. Reference strain *Lp* Paris 1 was used and is represented by a black node. Nodes are colored according to the result of sequence-based typing STs.
Lp, L. pneumophila; ST, sequence type.

ter understand the diversity and the distribution of clinical isolates; therefore, the Belgian NRC has recently added cgMLST to its panel of routine analyses [6].

As previously described, ST47 harbors few ADs in the 45 studied isolates. This could either be due to a very slow evolution rate and a lack of efficiency of the cgMLST scheme to discriminate this ST or to the persistence of a successful clone through time. ST47 is seldom retrieved from environmental investigations in water samples and soil has been suspected as contamination source [29]. It could explain why this ST's prevalence is limited to close geographical areas, such as the north and east of France, Belgium, Netherlands and south UK [22,29]. On the other hand, ST1 is challenging when comparing isolates from potential sources and clinical cases due to its dormancy state and slow pace genetic evolution, but it harbors more diversity in the cgMLST analysis. Two clusters of seven isolates each were highlighted, one already suspected previously to be caused by a clone reappearing over time and the other formed by epidemiologically unrelated cases [6] (Figure 4b). This goes along with previous observations that despite highly discriminant capacities of WGS analysis,

Table 1
Punctual mutations leading to resistance toward antibiotics that were screened for all isolates based on the literature evidence.

Gene	encoded Protein	Targeted Antibiotic	Reported mutations	Level of resistance	Presence in our dataset	Presence of close mutations in protein sequence	Reference species	Literature Reference
gyrA	DNA Gyrase subunit A	FQ	T83K T83I D87N D87H	Low Undetermined[1]	No No No No	No	*L. pneumophila*	Jonas 2003, Hennebique 2017 [28,29]
gyrB	DNA Gyrase subunit B	FQ	N426 K464 K447E 425 450 453 499 501	Low Undetermined[1]	No No No No No No No No	No No	*E. coli* *L. pneumophila*	Bhatnagarl & Wong 2019; Almahmoud 2009 [33,34] Uniprot · F8U7P3_LEGPN
parC	subunit of DNA topoisomerase IV	FQ	45 81 83 125 126	Undetermined[1]	No No No No No	L133P;L133H	*L. pneumophila*	Uniprot · Q5ZRA3_LEGPH
rpoB	beta-subunit of bacterial RNA polymerase	Rifampicin	Q528L Q528K N531R S537F H541Y R544H	Low to High	No No No No No No	No	*Legionella* non *pneumophila*	Nielsen 2000 [14]
rplD	50S ribosomal subnit L4	Macrolides	G66D G66S G66A G66R T65K del63KC64	Low	No No No No No No	No	*L. pneumophila*	Descours 2017 [13]
rplV	50S ribosomal subnit L22	Macrolides	G91D + P87L	Low	No	No mutations	*L. pneumophila*	Descours 2017 [13]
rrl	23S rRNA	Macrolides	G2051A A2052T,C,G A2053C,G C2605A,G,T	Low to High[2]	No Yes No No	No	*L. pneumophila*	Ginevra 2022; Descours 2017 [12,13]

FQ, Fluoroquinolones; rRNA, ribosomal RNA.
[1] Based on location of DNA binding-site and Quinolone Resistance-Determining Regions
[2] According to number of copies of 23S rRNA mutated and association with rplD or rplV mutations

the epidemiologic link always needs to be considered [6,8]. ST23, common in France and Italy, represents only 3.4% of our cases [5], which were closely related, as previously described [5]. Altogether, the data from the cgMLST show that this tool is generally more discriminant, and it will be used as a complementary tool for source investigations of the NRC, despite its limitations with ST1 and ST47.

All ST1 and related STs harbored the LpeAB efflux pump, which confers moderately elevated MICs to erythromycin, azithromycin, and spiramycin [10,30]. In Belgium, the first line treatment is clarithromycin, which remains efficient despite the presence of this pump. One isolate (LEG1112) harbored a high-level resistance to macrolides first identified by the presence of *rrl* gene A2052G mutation and subsequently confirmed by high MIC values and long-read WGS confirmation of short-read data. High-level resistance toward C14-15 macrolides azithromycin, erythromycin, and clarithromycin (>1024 mg/l) was previously related to *rrl* mutation in all three copies [10], which was confirmed by our data. The MIC for C16 josamycin remained moderately increased (8 mg/l). To the best of our knowledge, this is the first cultured clinical *Lp* isolate highly resistant to C14-C15 macrolides; it was responsible for a fatal LD case in 2019 for a patient treated initially by clarithromycin and later switched to levofloxacin. This retrospective and unexpected discovery highlights the need to establish standardized testing for AST and establish resistance breakpoints [13]. Genomic screening for antimicrobial resistance should be routinely performed for each clinical isolate and when AST is unavailable. However, our results show that analysis of short-read assembly is hindered for *rrl* screening because it exists as three genomic copies, but this can be overcome using hybrid assembly incorporating long-read WGS or creation of bioinformatical tools to overcome misalignment of multiple copy genes.

After further research, no epidemiologic link could be demonstrated between LEG1112 and the French isolates because the Belgian patient did not travel before the incubation period and no environmental source was related to his case. However, the whole genome SNP analysis validated a close genetic relatedness between the isolates, letting us fear the circulation of a ST that may have successfully acquired resistance, of which the triggering mechanism and frequency are not yet determined. It has been reported that macrolide resistance in *Lp* could be provoked *in vitro* by adding increasing concentrations of either erythromycin or azithromycin [11]. The appearance of this *in vivo* macrolide's resistance remains difficult to understand so far. A deeper investigation of genomic backgrounds of ST188 isolates should be performed to highlight other genetic and phenotypic differences and evaluate the potential for transmission or occurrence of such resistant *Lp*.

In conclusion, the increase of LD cases is of major concern because outbreak prevention is not always effective. All diagnostic tests remain of importance, including PCR protocols able to detect and diagnose non-SG1 and non-*pneumophila* species infections and serology due to its large testing capacity. The addition of WGS to our routine arsenal allows more extensive typing of isolates with cgMLST and better resolution to identify the potential circulation of individual clones, despite the absence of an obvious outbreak. Resistance toward antibiotics, although scarce, is concerning because the same resistant ST188 was found in Belgium and France. It should be monitored by genotypic investigation in the absence of available AST breakpoints.

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Ethics

This study was submitted and reviewed by the local ethical committee of the Universitair Ziekenhuis Brussel.

Declarations of competing interest

The authors have no competing interests to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2025.107786.

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