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Phenotypic and genotypic antimicrobial resistance of *Listeria monocytogenes*: an observational study in France



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Summary

Background Large-scale studies are needed to clarify antimicrobial resistance in the foodborne pathogen *Listeria monocytogenes* (*Lm*) and the effectiveness of listeriosis treatment options. Here we examined the antimicrobial resistance patterns in *Lm* over time and assessed genotype-phenotype concordances.

Methods We analyzed 5339 *Lm* isolates (2908 clinical and 2431 food isolates) collected in France and overseas territories, between 2012 and 2019. Whole genome sequencing was performed for all isolates and antimicrobial resistance profiles inferred from draft assemblies. Antimicrobial susceptibility towards 22 antimicrobials was determined for all clinical isolates, and in food isolates with acquired resistance genes.

Findings All tested isolates were resistant to at least 3 different classes of antimicrobials, consistent with *Lm* intrinsic traits. Acquired antimicrobial resistance in *Lm* was rare (2.23% isolates) and more prevalent in food (mainly lineage II) compared to clinical isolates (mainly lineage I) (3.74% vs 0.98%, $p < 0.0001$), and in isolates with disinfectants or stress resistance traits (e.g. *bcrABC*, 20.20% vs 7.20%, $p < 0.0001$), suggesting co-selection of resistance in food-production environments. Acquired antimicrobial resistance could be predicted from genomes with high accuracy (>99%), except for ciprofloxacin. Acquired antimicrobial phenotypes were towards tetracyclines (mostly due to *tetM*), trimethoprim (*dfpD*), lincosamides (*lnuG*), macrolides (*ermB*, *mphB*) and phenicols (*fexA*).

Interpretation The reference treatment for listeriosis (aminopenicillins/aminoglycosides) remains effective, with no acquired resistance observed. Continuous surveillance of antimicrobial resistance in clinical and food isolates is crucial to detect the emergence of novel resistance.

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Keywords: Listeriosis; Antimicrobial resistance; Genome; Phenotype; Prediction

Introduction

Listeria monocytogenes (*Lm*) is a Gram-positive facultative intracellular bacterium and a major foodborne pathogen. It causes listeriosis, a rare but deadly infection in humans and animals, that can manifest as mild gastroenteritis, bacteraemia, maternal-neonatal infections, or meningo-encephalitis.^{1,2} The incidence

of listeriosis has been increasing worldwide, with high mortality rates for most severe forms, despite antimicrobial treatment. The reference treatment for listeriosis relies on β -lactams (specifically aminopenicillins, like ampicillin or amoxicillin), usually combined with an aminoglycoside (most frequently gentamicin),³ to benefit from its synergistic bactericidal effect. In

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Research in context

Evidence before this study

We searched PubMed on 29 March 2023 for English-language studies on *Listeria monocytogenes* (*Lm*) antimicrobial resistance (AMR) published since 1 January 1980 using the keywords 'Listeria', 'listeriosis', 'food', 'antibiotic', 'antimicrobial' and 'resistance'. Studies could include human, food, animal and environmental data and had to include phenotypic and/or genomic analysis of antimicrobial susceptibility. The available studies either focus on a small number of isolates, in selected locations, over short periods of time, or provide large retrospective heterogeneous phenotypic-based data; in both cases, the heterogeneity and non-exhaustiveness of the study designs make it difficult to draw firm conclusions about AMR in *Lm* species. Furthermore, the concordance between AMR phenotype and genotype predictions has never been evaluated for *Lm*.

Added value of this study

This study is based on (i) a national surveillance system with almost complete coverage of isolates from microbiologically

reported cases (>98%) and food alerts (>80%) in France, and (ii) the unique combination of phenotypic and genotypic detection of AMR at the national level. We show that the rate of acquired AMR among isolates remains low. All clinical and food isolates were found to be susceptible to ampicillin/amoxicillin, the first line treatment for listeriosis. We also show that acquired AR in *Lm* can be inferred from genomic data with high accuracy (>99%).

Implications of all the available evidence

The results demonstrate that genomic analysis is a useful and accurate tool for predicting acquired *Lm* AR. They show that, contrary to unconfirmed published reports, the overall prevalence of acquired AMR is low and absent for aminopenicillins, demonstrating that the combination of amoxicillin/ampicillin and gentamicin (or co-trimoxazole if allergic) is a pharmacologically effective first-line treatment for invasive listeriosis.

patients allergic to β -lactams, cotrimoxazole (trimethoprim-sulfamethoxazole) is frequently used, sulfamethoxazole enhancing trimethoprim bactericidal activity even though *Lm* isolates exhibit sulfonamide resistance.⁴ The use of vancomycin, fluoroquinolones, rifampicin, and linezolid is also reported.^{5,6}

Lm is naturally resistant to certain antimicrobials, such as third generation cephalosporins (e.g. cefotaxime and ceftriaxone), monobactams (aztreonam) and oxacillins, primarily due to the absence of appropriate penicillin binding proteins (PBPs).³ It also demonstrates inherent resistance to first-generation quinolones (nalidixic acid), fosfomycin and sulfonamides,⁵⁻⁷ due to the presence resistance genes in its core genome. Occasionally, acquired resistances have been reported in *Lm*, as a result of gene acquisitions through horizontal gene transfer from enterococci-streptococci or mutations in core genes (for a review see Baquero and colleagues⁵). Specifically, acquired resistances in *Lm* have been observed following the acquisition of resistance genes encoding for ribosomal protection proteins (*tetM/tetS*), efflux proteins (*tetK/tetL*) conferring resistance to tetracycline, alternative dihydrofolate reductases (*dfrD/dfrG*) that circumvent the inhibitory effect of trimethoprim, 23S ribosomal RNA methyltransferases (*ermB*) that blocks binding of erythromycin, and chloramphenicol acetyltransferases (*catA*) that inactivate chloramphenicol by addition of an acyl group. Furthermore, mutations in *Lm* core genes that result in a reduced affinity of antimicrobials for their target enzyme have also been reported, leading to resistance to ciprofloxacin (*gyrAB/parC/lde* mutations), rifampicin (*rpoD*), or streptomycin (*rrm*).⁵

Although whole genome sequencing has become a widely accepted and routine typing tool for *Lm* surveillance, its application for predicting phenotypic antimicrobial susceptibility has not been proven in *Lm* isolates. Here we evaluated the prevalence of antimicrobial resistance in *Lm* clinical and food isolates collected in France and overseas territories, to investigate the concordance between genome-based predictions and disk diffusion tests and susceptibility trends over time.

Methods

Bacterial isolates

All clinical and food *Lm* isolates prospectively collected by the French National Reference Centre *Listeria* (Institut Pasteur, Paris, France) were considered for this study. The initial dataset comprised 3205 clinical isolates collected between 2012 and 2019 and 5428 food isolates collected between 2015 and 2019. All isolates were subject to whole genome sequencing (see below) and strain typing using the *Lm* core genome multilocus sequencing typing (cgMLST) scheme of 1748 core genes.⁸ To avoid duplication, only one isolate of the same patient, food sample, food alert or industry own-check number was kept for downstream analysis, if they belonged to the same cgMLST type.⁸ After the deduplication process, the final dataset analysed consisted of 5339 *Lm* isolates (2908 clinical and 2431 from food).

Clinical isolates were obtained from cases of bacteraemia ($n = 1,629$, 56%), neurolisteriosis ($n = 746$, 26%), maternal-neonatal infections ($n = 295$, 10%) and other types of invasive infections ($n = 238$, 8%). Food-

associated isolates were obtained from food alerts (defined as investigations on foodstuffs not complying microbiological criteria for *Lm*, according to the European Commission (EC) regulation EC 2073/2005) ($n = 1,996$, 82%), food investigations around neuro-listeriosis cases ($n = 93$, 4%) and food industries own-checks ($n = 342$, 14%). Isolates were from mainland France ($n = 2853$ clinical, $n = 2424$ food) and overseas territories ($n = 55$ clinical, $n = 7$ food).

Bacterial species identification was performed using the MALDI-TOF mass spectrometry MicroFlex LT system (MBT library DB-7854, Bruker Daltonics, Germany) as described.⁹

Genome sequencing and analyses

Genomic DNA was extracted using either the DNeasy Blood and Tissue Extraction kit (Qiagen, Denmark) or the NucleoSpin Tissue purification kit (Macherey–Nagel, Düren, Germany) from 0.9 mL Brain heart infusion (BHI, Difco, France) cultures grown overnight at 35 °C. DNA libraries were prepared using the Nextera XT DNA Sample kit (Illumina, USA) and sequenced with Illumina NextSeq 500 using a 2 × 150 bp paired-end run. Short raw reads were trimmed with fqCleaner v.21.1 ([gitlab.pasteur.fr/GIPhy/fqCleanER/](https://github.com/pasteur.fr/GIPhy/fqCleanER/)) as previously described⁸ and assembled with unicycler v.0.4.8, which uses SPAdes/3.14.0 with a wide range of k -mer sizes, improving assembly accuracy and reducing the rate of misassembly (<https://github.com/rrwick/Unicycler>). Contigs smaller than 300 bp were discarded. *In silico* strain typing was performed from the draft assemblies using BIGSdb-*Lm* (<https://bigsdbs.pasteur.fr/listeria/>)^{8,10} as previously described.⁸ Assemblies were screened for the presence of antimicrobial resistance genes with ABRicate v.1.0.1 (<https://github.com/tseemann/ABRicate>) using NCBI AMRFinderPlus,¹¹ CARD,¹² Resfinder,¹³ ARG-ANNOT¹⁴ and BIGSdb-*Lm*,⁸ to include general and genus-specific databases. Point mutations in core genes leading to amino acid substitutions were inspected in MAFFT v.7.467¹⁵ protein sequence alignments.

To infer on the genetic location of acquired resistance genes, representatives of each acquired genotype were selected for nanopore long-read sequencing (Oxford Technologies, UK). DNA libraries were prepared using the Rapid Sequencing Kit and sequenced on MinION Mk1C sequencer (software MinKNOW 21.11.6) with FLO-MIN106D flow cells (R9.4.1 chemistry). Long reads were assembled with unicycler v.0.4.8 (<https://github.com/rrwick/Unicycler>) and screened for the presence of plasmids, phages and insertion sequences in the context of acquired genes using MOB-suite v.2.0.1¹⁶, PHASTER (<https://phaster.ca/>)¹⁷ and ISEScan,¹⁸ respectively.

Antimicrobial susceptibility testing

Clinical isolates ($n = 2908$) were subjected to phenotypic antimicrobial susceptibility testing using the disk

diffusion method according to the guidelines of EUCAST.¹⁹ All food isolates with detected genetic traits of acquired resistance ($n = 89$) were also tested. The inhibition zone diameters were measured for the following 22 antimicrobials: amoxicillin (AMX, 25 µg), ampicillin (AMP, 10 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), clindamycin (CLI, 2 µg), chloramphenicol (CHL, 30 µg), erythromycin (ERY, 15 µg), fosfomycin (FOS, 50 µg), fusidic acid (FUS, 10 µg), gentamicin (GEN, 15 µg), imipenem (IPM, 10 µg), tetracycline (TET, 30 µg), levofloxacin (LVX, 5 µg), moxifloxacin (MXF, 5 µg), nalidixic acid (NAL, 30 µg), kanamycin (KAN, 30 µg), penicillin G (PEN, 6 µg), rifampicin (RIF, 30 µg), streptomycin (STR, 10 µg), sulfonamides (SUL, 200 µg), trimethoprim (TMP, 5 µg), and vancomycin (VAN, 30 µg) (Bio-Rad, France). Clinical isolates carrying *aacA4/aac(6)-Ib3* genes ($n = 4$) were also tested for amikacin (AMK, 30 µg) and tobramycin (TOB, 10 µg). In the absence of internationally standardized *Lm* breakpoints for the antimicrobials and/or concentrations tested, the number of resistant isolates were considered using: *i*) disk contact resistance (i.e. diameter of growth inhibition halo of 6 mm) and *ii*) general breakpoints criteria based on the pharmacokinetics and pharmacodynamics (PK/PD) of antimicrobials, proposed by the *Société Française de Microbiologie*.²⁰ *Lm* epidemiological cut-off values (ECOFFs) were identified according to EUCAST,²¹ based on the distributions of inhibition zone diameters determined in this study, to distinguish between isolates with and without acquired resistance mechanisms. All breakpoints used are detailed in Table 1.

Minimal inhibitory concentrations (MICs) were determined by E-test (bioMérieux, France) according to manufacturer instructions, for isolates with acquired resistance genes ($n = 119$).

Statistical analysis

Statistical analysis was conducted to investigate the relationship between the year of collection and the inhibition zone diameter for each antimicrobial. Initially, histograms and best-fitted density plots were employed to assess the Gaussian distribution of diameters. A linear regression model with categorical year was used. To ensure the validity of the linear regression model, the Gaussian distribution of the residuals were examined, and homoscedasticity was validated by plotting the residuals against the predicted values, fitting a linear prediction line to the scatter plot, and confirming that the residuals' slope was not significantly different from 0. The residuals exhibited a Gaussian distribution, and the homoscedasticity assumption was satisfied for all antimicrobials, except for CTX, FOS, NAL, and SUL (intrinsic resistances, non-Gaussian distributions; Fig. 1), for which assumptions for linear regression models were violated.

Antibiotic (disk content)	Contact resistance Breakpoints		PK/PD breakpoints		Minimum diameter in isolate with no resistance (mm)	ECOFF	
	Breakpoint (R = mm)	No. resistant isolates (%; n = 2908)	breakpoint (R < mm)	No. resistant isolates (%; n = 2908)		Breakpoint (R < mm)	No. resistant isolates (%; n = 2908)
AMP (10 µg)	6	0 (0%)	16	0 (0%)	20	16	0 (0%)
AMX (25 µg)	6	0 (0%)	16	0 (0%)	22.8	16	0 (0%)
PEN (6 µg)	6	0 (0%)	18	0 (0%)	16.4	16	0 (0%)
CTX (30 µg)	6	1993 (69%)	23	2869 (99%)	n.a.	n.a.	n.a.
IPM (10 µg)	6	0 (0%)	17	0 (0%)	23.1	17	0 (0%)
GEN (15 µg)	6	0 (0%)	16	0 (0%)	17.2	16	0 (0%)
KAN (30 µg)	6	0 (0%)	10	0 (0%)	11	10	0 (0%)
STR (10 µg)	6	0 (0%)	13	263 (9%)	9	9	0 (0%)
ERY (15 µg)	6	1 (0%)	17	2 (0%)	14.5	14	1 (0%)
NAL (30 µg)	6	2898 (100%)	15	2906 (100%)	n.a.	n.a.	n.a.
CIP (5 µg)	6	4 (0%)	22	1316 (45%)	12.3	12	4 (0%)
LXV (5 µg)	6	0 (0%)	17	9 (0%)	12	12	0 (0%)
MXF (5 µg)	6	0 (0%)	21	47 (2%)	13	13	0 (0%)
TET (30 µg)	6	15 (1%)	17	26 (1%)	13.8	13	25 (1%)
RIF (30 µg)	6	0 (0%)	14	0 (0%)	14.6	14	0 (0%)
CLI (2 µg)	6	216 (7%)	15	1869 (64%)	n.a.	n.a.	n.a.
CHL (30 µg)	6	0 (0%)	19	60 (2%)	15	15	0 (0%)
SUL (200 µg)	6	2511 (86%)	12	2532 (87%)	n.a.	n.a.	n.a.
TMP (5 µg)	6	0 (0%)	10	0 (0%)	23	10	0 (0%)
FOS (50 µg)	6	2670 (92%)	14	2792 (96%)	n.a.	n.a.	n.a.
VAN (30 µg)	6	0 (0%)	17	1 (0%)	13.3	13	0 (0%)
FUS (10 µg)	6	0 (0%)	15	0 (0%)	15	15	0 (0%)

Abbreviations: PK/PD, pharmacokinetics and pharmacodynamics antimicrobial breakpoints,²⁰ n.a., not applicable (intrinsic resistance); ECOFF, epidemiological cut-off, obtained in this study according to EUCAST guidelines.²¹ Values in bold highlight adjusted PK/PD cut-offs based on the natural susceptibility distributions observed in this study.

Table 1: Number of resistant *L. monocytogenes* isolates (2012–2019, n = 2908 clinical isolates) and interpretative breakpoints used for each antibiotic.

The prevalence of acquired resistance loci among distinct isolates groups were assessed with chi-square test or Fisher’s exact test when required. Logistic regression model was used to estimate the odds ratio and the 95% confidence interval; the firthlogit correction was used in case the proportion was 0 in one group.

Genotypic and phenotypic antimicrobial resistance concordance

The concordance between phenotypic (P) and genotypic (G) traits of resistance was calculated for each antimicrobial as:

$$\text{Concordance (in \%)} = \frac{(P1G1+P0G0)}{(P0G1+P1G0+P1G1+P0G0)} \times 100$$

where P1G1 refers to true positives (i.e. the phenotypic and genotypic traits are simultaneously present), P0G0 refers to true negatives (phenotypic and genotypic traits are simultaneously absent), P1G0 denotes false negatives (phenotype present but no genotype of resistance detected) and P0G1 denote false pheno-negatives (genotype present but phenotype of resistance absent).

The sensitivity (i.e. accuracy of G1 to predict P1) and specificity (i.e. accuracy of G0 to predict P0) of genotypic predictions were calculated as:

$$\text{Sensitivity (in \%)} = \frac{P1G1}{(P1G1+P1G0)} \times 100$$

$$\text{Specificity (in \%)} = \frac{P0G0}{(P0G0+P0G1)} \times 100$$

Role of the funding source

The funders of the study had no role in study design, data analysis, data interpretation, or writing.

Results

Phenotypic and *in silico*-predicted antimicrobial resistance in clinical isolates

Clinical isolates (n = 2908) were distributed into 3 phylogenetic lineages (lineage I, n = 1,804, 62%; II, n = 1103, 38%; and III, n = 1) and 93 cgMLST sub-lineages, mainly represented by SL1 (n = 536, 18%), SL6 (n = 333, 12%), SL4 (n = 305, 11%) and SL2 (n = 152, 5%), as previously reported.²² A total of 2042 genotypes (cgMLST cut-off of 7 or less alleles out of 1748 loci) were observed: 2035 were country-specific and 7 (0.3%) were present in both mainland and overseas territories (Réunion, New Caledonia and/or Guadeloupe). No common genotypes were observed between mainland and the other overseas territories (French Polynesia, French Guiana, Mayotte and Martinique).

When considering stringent breakpoints (contact resistance), most isolates were resistant to at least 4

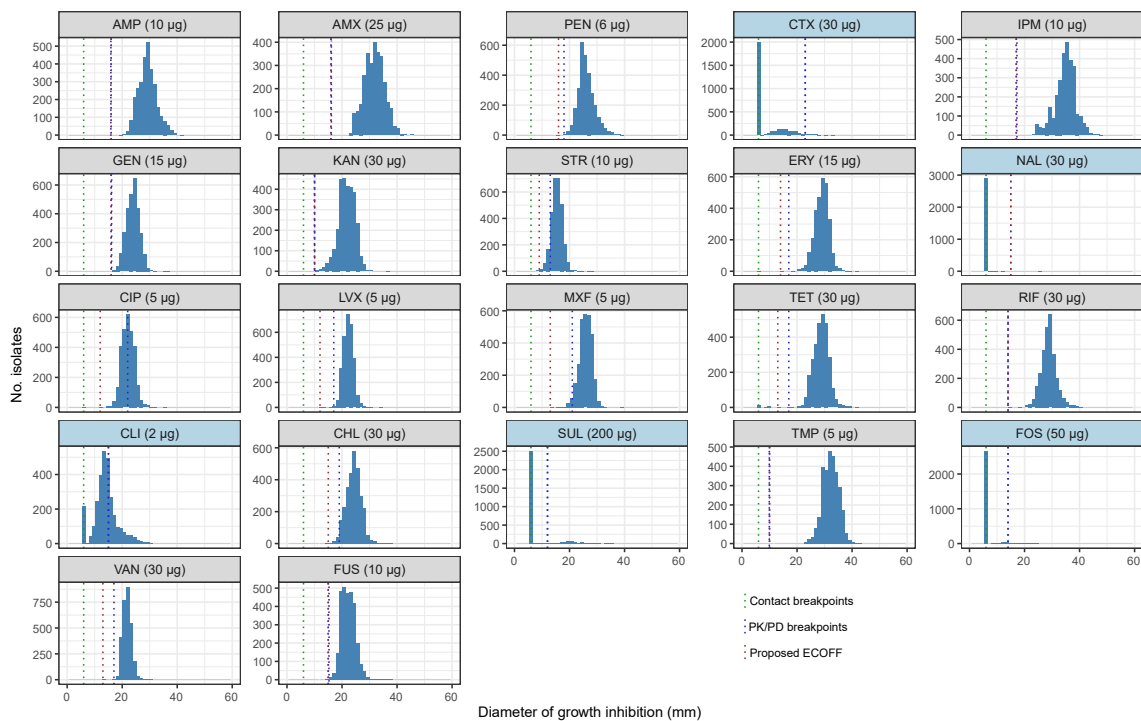


Fig. 1: Distribution of the diameters of growth inhibition (halos) for 22 antimicrobials (2012–2019, $n = 2908$ clinical isolates). Plots are ordered by antimicrobial class: beta-lactams (AMP, ampicillin; AMX, amoxicillin; PEN, penicillin G; CTX, cefotaxime; IPM, imipenem), aminoglycosides (GEN, gentamicin; KAN, kanamycin; STR, streptomycin), macrolides (ERY, erythromycin), quinolones (NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin); tetracyclines (TET, tetracycline), rifamycins (RIF, rifampicin), lincosamides (CLI, clindamycin), phenicols (CHL, chloramphenicol), sulfonamides (SUL, sulfonamides); folate-inhibitors (TMP, trimethoprim); others (FOS, fosfomycin; FUS, fusidic acid; VAN, vancomycin). The antimicrobial disk content is shown in brackets. Antimicrobial names on light blue boxes highlight non-Gaussian distributions (intrinsic resistance). Dotted lines denote resistance breakpoints: green, contact resistance (diameter = 6 mm); blue, general PK/PD breakpoints²⁰; red, adjusted epidemiologic cut-offs for natural susceptibility distributions (ECOFF).

antimicrobials (*Lm* intrinsic resistance): NAL ($n = 2898$, 100%), FOS ($n = 2670$, 92%), SUL ($n = 2511$, 86%), CTX ($n = 1993$, 69%). Few were contact-resistant to CLI ($n = 216$, 7%), CIP ($n = 4$, 0%), TET ($n = 15$, 1%) and ERY ($n = 1$, 0%). Notably, no contact resistance was observed for 14 antimicrobials (AMP, AMX, PEN, IPM, GEN, KAN, STR, LVX, MXF, RIF, CHL, TMP, VAN and FUS) and the diameters' distributions for those antimicrobials followed a normal distribution (Fig. 1, Table 1).

Similarly, when considering general PK/PD breakpoints (Fig. 1, Table 1), most isolates were resistant to NAL ($n = 2906$, 100%), FOS ($n = 2792$, 96%), SUL ($n = 2532$, 87%), CTX ($n = 2869$, 99%) (*Lm* intrinsic resistances) and none were resistant to AMP, AMX, PEN, IPM, GEN, KAN, RIF, TMP, FUS. However, an overestimation of resistant isolates was observed to CLI ($n = 1869$, 64%), CIP ($n = 1316$, 45%) ERY ($n = 2$, 0%), STR ($n = 263$, 9%), CHL ($n = 60$, 2%), MXF ($n = 47$, 2%), TET ($n = 26$, 1%), LVX ($n = 9$, 0%) and VAN ($n = 1$, 0%). Although some antimicrobials (CHL, CIP, CLI, MXF, STR) did not fit the diameter's distributions, PK/PD

breakpoints were appropriate for first-line antimicrobials (AMX, AMP, PEN, TMP, GEN), confirming the susceptibility of our isolates to these major antimicrobials.

Resistance genetic traits included the core genes *fosX* (*lmo1702*), *norB* (*lmo2818*), *lin* (*lmo0919*), *sul* (*lmo0224*) and 10 *pbp* genes. Acquired resistance genes (Table 2) were present in 30 clinical isolates (1.03%, all from mainland France), and were associated with resistance towards tetracyclines (*tetM*, $n = 25$; *tetL*, $n = 1$; *tetK*, $n = 1$) and erythromycin (*ermB*, $n = 1$). Genes conferring resistance to aminoglycosides (*aacA4*, $n = 4$) were also detected, but were not associated with any detectable phenotypic resistance to the aminoglycosides tested (STR, GEN, KAN, AMK and TOB). Overall, when using stringent resistance breakpoints (Table S1), average genotypic and phenotypic concordance was of 89%, 71% for intrinsic resistance and of 99.97% for acquired traits except for CIP (0.7%). Using the general PK/PD breakpoints (Table S1), average concordance was of 94%, 89% for intrinsic resistance and of 99% for acquired traits except for CIP (45%), with no genetic basis

Acquired genotype ^a	No. isolates (%; N = 5339)	No. genotypes (%; N = 3290)	Genomic location or genetic context	Diameter of inhibition halo (mm)	MIC (µg/ml)	Source ^b (no. isolates)	Sublineage (Clonal Complex)	
							Lineage I	Lineage II
<i>aacA4</i> (<i>aac</i> (6')-Ib3)	49 (0.92%)	34 (1.03%)	Prophage	GEN: 21.3–28.6; KAN:20.5–26.5; STR: 13.9–17.6	n.d.	H (4), F (45)	SL3(CC3), SL5(CC5), SL9(CC9), SL31(CC31), SL312(CC31)	
<i>aphA</i> (<i>aph</i> (3')-IIIa)	10 (0.19%)	4 (0.12%)	Plasmid-borne transposon	GEN: 25.5–30.6; KAN:24.1–26.6; STR: 15.8–19.4	n.d.	F (10)	–	SL313(CC31)
<i>dfrD</i> , <i>lnuG</i> , <i>mphB</i>	1 (0.02%)	1 (0.03%)	Plasmid	TMP: 6.0; CLI: 13.7; ERY: 30.8	TMP: >32; CLI: 1.5; ERY: 0.25	F (1)	–	SL325(CC31)
<i>ermB</i>	5 (0.09%)	5 (0.15%)	Inc18 plasmid	ERY: 6.0–32.4	ERY: 0.125–>256	H (1), F (4)	SL4(CC4), SL386(CC388), SL5(CC5)	–
<i>fexA</i>	3 (0.06%)	2 (0.06%)	Chromosomal recombinase XerC	CHL: 10.7–25.0	CHL: 4–32	F (3)	–	SL14(CC14), SL415(CC415)
<i>tetM</i>	41 (0.77%)	23 (0.70%)	Transposon Tn916	TET: 6.0–31.0	TET: 0.125–64	H (19), F (22)	SL1(CC1), SL4(CC4), SL5(CC5), SL59(CC59)	SL399(CC14), SL18(CC18), SL199(CC199), SL20(CC20), SL7(CC7), SL16(CC8), SL9(CC9), SL91(CC14), SL11(CC11), SL325(CC31)
<i>tetM</i> , <i>tetL</i>	10 (0.19%)	2 (0.06%)	Transposon Tn916	TET: 6.0–10.2	TET: 48–64	H (5), F (5)	–	SL14(CC14)
<i>tetM</i> , <i>tetK</i>	1 (0.02%)	1 (0.03%)	Transposon Tn916	TET: 13	TET: 32	H (1)	SL5(CC5)	–

^aGene traits of antibiotic resistance towards aminoglycosides (*aacA4*, *aphA*), folate inhibitors (*dfrD*), lincosamides (*lnuG*), macrolides (*ermB*, *mphB*), phenicols (*fexA*), tetracyclines (*tetM*, *tetL*, *tetK*). ^bSource: H, human; F, food.

Table 2: Acquired antibiotic resistance genotypes and corresponding phenotypes detected in this study (119 *Listeria monocytogenes* isolates out of 5339).

found for the overrepresentation of STR, CHL, MXF, LVX, PEN, VAN, highlighting the need to use appropriate epidemiological cut-offs for *Lm*.

No increase of resistance was observed over time from 2012 to 2019 (Figure S1, Tables S2–S4). Logistic regression models employed, using 2014 as an arbitrary reference year, showed an overall significant increase of inhibition diameters (i.e. higher susceptibility) observed after 2017 (Figure S1, Table S4), which could potentially be explained by methodological changes (e.g. improved quality of antimicrobial disks; improved precision of automated measurement systems).

In silico-prediction of antimicrobial resistance in food isolates

Given the high genotypic and phenotypic concordance observed for acquired resistance (>99%, except for CIP) in clinical isolates, we then looked at the presence of resistance traits in 2431 genomes of food isolates. Those were distributed into 3 phylogenetic lineages (lineage I, *n* = 634, 26%; lineage II, *n* = 1796, 74%; lineage III, *n* = 1) and 77 sublineages. Most prevalent sublineages in food isolates were SL121 (*n* = 586, 24%) and SL9 (*n* = 331, 14%), as previously reported in France.²² A total of 1523 genotypes (cgMLST cut-off of 7 or less alleles out of 1748 loci) were observed, none present in both mainland and overseas territories.

Intrinsic genes *fosX*, *norB*, *lin*, *sul* and *pbp* genes were present in all isolates. Acquired resistance genes were detected in 89 isolates (3.66%, all from mainland France, except isolate LM201902657, cgMLST L2-SL31-ST31-CT6032, from Réunion), resulting in reduced inhibition diameters (Table 2, Table S5) towards tetracyclines (*tetM*, *n* = 27; *tetL*, *n* = 5), macrolides (*ermB*, *n* = 4; *mphB*, *n* = 1), phenicols (*fexA*, *n* = 3), folate inhibitors (*dfrD*, *n* = 1) and lincosamides (*lnuG*, *n* = 1), but not towards aminoglycosides (*aacA4*, *n* = 45; *aphA*, *n* = 10). One isolate (LM201900136, from pork sausage, cgMLST type L2-SL325-ST325-CT5076) harboured *dfrD*, *lnuG*, *mphB* and *tetM* genes (disk susceptibility TMP: 6 mm, TET: 10.5 mm, CLI: 13.7 mm) (Table S5). The prevalence odds ratio (OR) of acquired antimicrobial resistance genes were significant lower in clinical isolates than in food isolates for the same period (2015–2019, 0.98% vs 3.66%, respectively; OR 0.26; 95% CI, 0.15–0.44, *p* < 10⁻⁴).

Genetic context of acquired resistance

Acquired resistance genes were located either within transposons, prophages or plasmids (Table 2). LM201900136 harboured simultaneously chromosomal (*tetM*) and plasmid-borne (*dfrD*, *lnuG*, *mphB*) acquired resistance genes. No known mutations associated to antimicrobial resistance phenotypes were detected in

rpoB/lmo0258, *gyrA/lmo0007*, *gyrB/lmo0006*, *parC/lmo1287*, *parE/lmo1289* or *lde/lmo2741* core genes.

Discussion

The emergence and spread of antimicrobial-resistance is a global concern, particularly in bacteria that can be transmitted to humans through the food chain. Previous studies have evaluated whole genome sequencing as a routine tool for monitoring antimicrobial resistance in *Salmonella enterica* Typhimurium, *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium*, demonstrating high concordance (99.74%) between phenotypic and predicted antimicrobial susceptibility.²³ Here we assessed the prevalence of antimicrobial resistance in clinical and food *Lm* isolates from France and overseas territories and evaluated the prediction of resistance from genome assemblies.

The dataset here analysed confirmed the previously described diversity of *Lm* in France,²² with a prevalence of lineage I in clinical isolates and lineage II in food isolates. The prevalence of acquired resistance (1.03% in clinical and 3.66% in food isolates) was also not statistically different from French studies performed in the previous decade [1.2%²⁴ and 2.5%,²⁵ respectively]. No acquired antimicrobial resistance transmission between mainland and overseas French territories was observed. However, this finding may be attributed to the relatively small number of *Lm* isolates obtained from those regions in the context of national surveillance for the period of the study ($n = 55$ clinical, $n = 7$ food).

Lm antimicrobial susceptibility remained overall stable over the 8-year study period (Figure S1; Table S4). Earlier studies conducted in other countries such as Denmark,²⁶ the UK²⁷ and Spain,²⁸ have consistently reported no differences in *Lm* susceptibility over time, despite variations in geographical locations, sampling periods and sources and strain types. As expected, isolates were naturally resistant to multiple classes of antimicrobials, either due to the lack of antimicrobial target (CTX) or to the presence of core genome resistance genes (FOS, CLI, NAL, SUL). However, the regulation of intrinsic resistance genes in *Lm* is highly complex and phenotypes are difficult to predict.²⁹ Intrinsic resistance to cephalosporins (CTX pheno-genotypic concordance of 69%) is due to its low affinity binding to *Lm* PBPs³ although several regulators of gene expression (such as *mdrL/lmo1209*, *oatA/lmo1291* and *lisR/lmo1377*) and mutations in *pbpB3/lmo0441* and *pbpA2/lmo2229* can increase susceptibility to CTX.^{29–31} *Lm* also exhibit natural resistance to quinolones (NAL pheno-genotypic concordance of 99.6%) and sulfonamides (SUL pheno-genotypic concordance of 86.5%),³² probably through efflux pumps (*norB/lmo2818*) and dihydropteroate synthase (*sul/lmo0224*) genes, respectively. FOS intrinsic resistance mediated by the core gene *fosX/lmo1702* (pheno-genotypic concordance of

92%) can be epistatically modulated by virulence genes, increasing antimicrobial influx into the bacterial cell and FOS susceptibility.³³ The intrinsic resistance to lincosamides mediated by the expression of the core gene *lin/lmo0919* (CLI pheno-genotypic concordance of 7.4%) was shown to be modulated by the structural interplay of terminator/anti-terminator structures in the 5' UTR of this gene.³⁴ Thus, the low concordance between phenotypes and intrinsic genotypes can be explained by this complex gene regulation.

Importantly, we show here that acquired resistance remains a rare event for *Lm*, and that acquired resistance phenotypes can be predicted from genome sequences with a remarkable concordance (>99%), except for CIP (<46%). Fluoroquinolone resistance primarily arises from mutational alterations in target DNA gyrase genes (*gyrA* and *gyrB*), topoisomerase IV genes (*parC* and *parE*), or mutations in regulatory genes affecting efflux pumps (eg. *lde/lmo2741*). Here, no specific gene variants could be associated to the 4 CIP resistant isolates, thus leading to a lower phenotype-genotype concordance for this antimicrobial.³⁵ Nevertheless, the distribution of inhibition zones diameters observed likely corresponds to the natural susceptibility distribution within the *Lm* population and could be used to define epidemiological cut-offs (ECOFFs; Table 1 and red lines in Fig. 1).²¹

The most common acquired resistance phenotype in both clinical and food isolates from different clonal complexes was to tetracyclines, mainly due to the presence of Tn916-carrying *tetM* genes (Table 2). TetM ribosomal protection proteins were originally described in *Streptococcus* and have been reported in several other Gram-positive and Gram-negative bacterial species³⁶ including clinical *Lm* isolates³⁷ and *L. innocua* isolates from cattle farms using tetracyclines.³⁸ Here, information on antimicrobial treatment was available for 6 out of 25 clinical cases with isolates harbouring *tetM* and did not include any tetracycline prescription during *Lm* infection, suggesting that gene acquisition was not due to antimicrobial exposure in those patients and likely have occurred outside the host.

Acquired *ermB*, encoding for a 23S ribosomal RNA methyltransferase conferring macrolide-lincosamide-streptogramin antimicrobial resistance,³⁹ was previously reported in France in 2 food isolates (1% of 202 isolates).²⁵ Here *ermB* was present in 5 isolates (0.1%) from both clinical (central nervous system infection) and food environments in Inc18 plasmids, a large group of enterococcal/streptococcal plasmids with broad host range.⁴⁰ Prior antimicrobial exposure could not be determined.

Acquired *dfrD* has previously been reported in food and clinical *Lm* isolates in France.^{24,25} Here *dfrD* was present in a plasmid that also contained *lnuG* and *mphB*, in a food isolate (pork sausage) that carried a chromosomal Tn916 *tetM* gene (Table 2, Table S5). Acquired *cat*

genes or *rpoB* rifampicin-resistance mutations previously reported in France^{24,25,41} were not detected in this study.

Phenotypic resistance towards aminoglycosides, which are used synergistically with first-line aminopenicillins in listeriosis treatment, was not observed in isolates harbouring either *aacA4* ($n = 45$) or *aphA* ($n = 10$). Whether the extended spacing of the -10-promoter box (located at -48 and -50 nucleotides upstream of the first nucleotide of the start codon of *aacA4* and *aphA*, respectively) could dampen promoter activity and gene transcription will be addressed in future studies.

Our combined genotype-phenotype screening approach used both general and organism-specific databases. This allowed for a more comprehensive

detection of resistance traits, including *Lm*-specific genes not found in general databases (e.g. *sul/lmo0224*) and, conversely, genes rarely reported in *Listeria*, such as the plasmid-encoded chloramphenicol exporter *fexA* described in *Staphylococcus lentus*⁴² detected here in the vicinity of a chromosomal XerC recombinase (Table 2).

In addition to antimicrobial resistance, genome screening also identified other antimicrobial resistance traits present in these isolates (Fig. 2; Table 3). These included (i) the integral membrane protein gene *mprF*, which is also part of the *Lm* core genome, and confers resistance to cationic peptides and defensins, and (ii) acquired genes conferring resistance to benzalkonium chloride disinfectants or stress islands (Fig. 2; Table S5). Previous reports on *Lm* CC6 clinical isolates have associated the presence of plasmid-borne *emrC* gene to

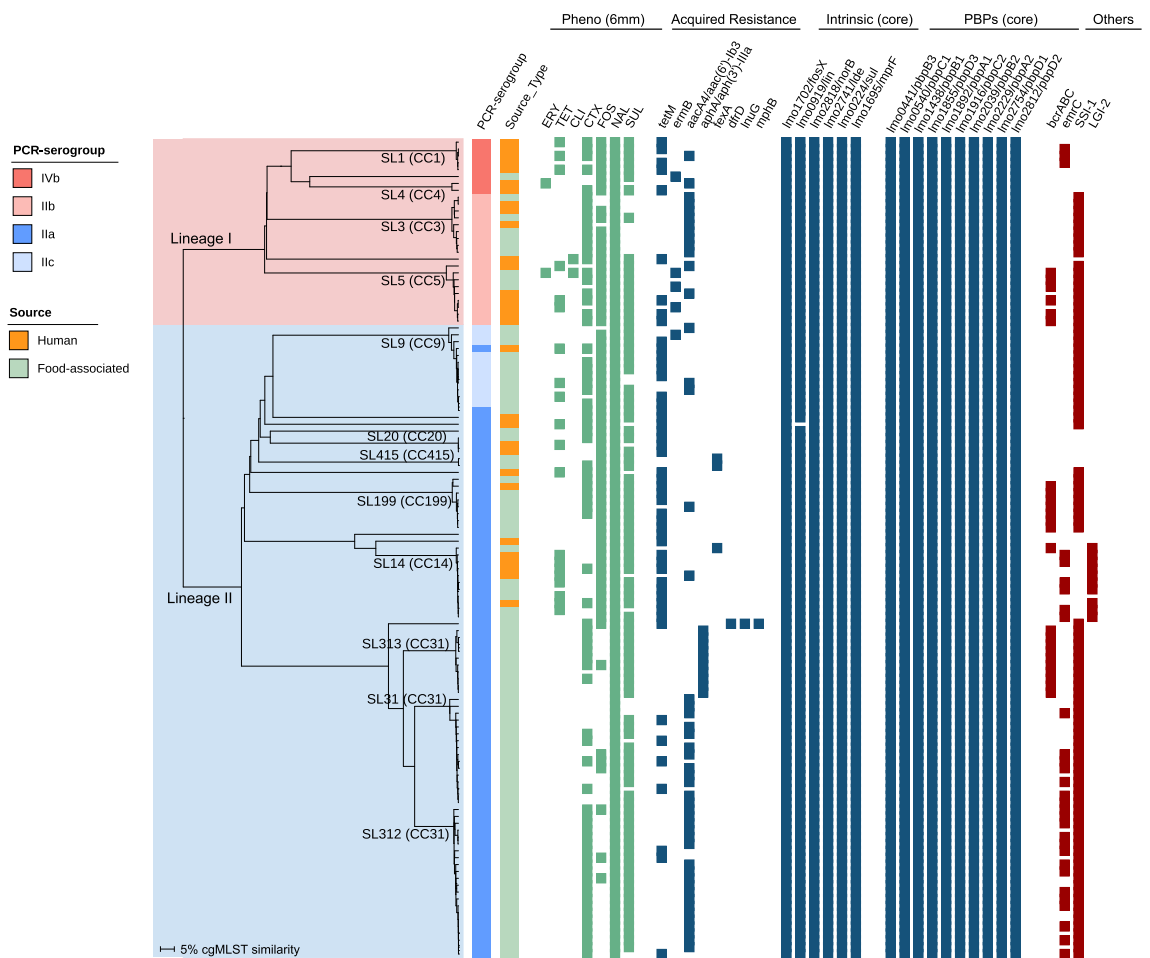


Fig. 2: Phylogenetic distribution of clinical and food *Listeria monocytogenes* isolates with acquired resistance traits (119 isolates out of 5339). Single linkage dendrogram based on cgMLST profiles (1748 loci, Institut Pasteur scheme). Lineages and sublineages with 2 or more isolates are labelled in the branches and corresponding clonal complexes are indicated in brackets. PCR-serogroups and source of isolation are colored as indicated in the key panels. Green boxes indicate isolates displaying antimicrobial resistance phenotypes (ERY, erythromycin; TET, tetracycline; CLI, clindamycin; CTX, cefotaxime; FOS, fosfomycin; NAL, nalidixic acid; SUL, sulfonamides). Blue boxes indicate isolates displaying antimicrobial resistance genes. Red boxes indicate isolates with disinfectants or stress resistance traits.

Locus	Description (reference)	Full dataset (N = 5339)	With acquired AR (n = 119)	Without acquired AR (n = 5220)	Odds Ratio (95% CI)	p-value AR vs non-AR ^a	Clinical (n = 2908)	Food-associated (n = 2431)	Odds Ratio (95% CI)	p-value H vs F ^b	Lineage I (n = 2438)	Lineage II (n = 2899)	Odds Ratio (95% CI)	p-value I vs II ^c
<i>bcrABC</i>	BC resistance ⁴³	401 (7.5%)	24 (20.2%)	377 (7.2%)	3.23 (2.04–5.26)	<0.0001	116 (4.0%)	285 (11.7%)	0.31 (0.25–0.39)	<0.0001	118 (4.8%)	283 (9.8%)	0.47 (0.38–0.59)	<0.0001
<i>qacA</i>	BC resistance ⁴⁴	70 (1.3%)	0 (0.0%)	70 (1.3%)	0.31 (0.02–5.00)	0.41	22 (0.8%)	48 (2.0%)	0.38 (0.23–0.63)	<0.0001	4 (0.2%)	66 (2.3%)	0.07 (0.03–0.19)	<0.0001
<i>emrC</i>	BC resistance ⁴³	313 (5.9%)	32 (26.9%)	281 (5.4%)	6.67 (4.17–10.00)	<0.0001	130 (4.5%)	183 (7.5%)	0.57 (0.46–0.72)	<0.0001	101 (4.1%)	212 (7.3%)	0.55 (0.43–0.70)	<0.0001
<i>Tn6188::emrC</i>	BC resistance ⁴⁵	843 (15.8%)	0 (0.0%)	843 (16.1%)	0.02 (0.001–0.35)	0.01	162 (5.6%)	681 (28.0%)	0.15 (0.13–0.18)	<0.0001	66 (2.7%)	777 (26.8%)	0.08 (0.06–0.10)	<0.0001
SSI-1	Stress island, low pH & high salt tolerance ⁴⁶	1869 (35.0%)	91 (76.5%)	1778 (34.1%)	6.25 (4.17–10.00)	<0.0001	833 (28.6%)	1036 (42.6%)	0.54 (0.48–0.61)	<0.0001	492 (20.2%)	1377 (47.5%)	0.28 (0.25–0.32)	<0.0001
SSI-2	Stress island, high pH & oxidative stress tolerance ⁴⁷	767 (14.4%)	0 (0.0%)	767 (14.7%)	0.02 (0.002–0.39)	0.01	136 (4.7%)	631 (26.0%)	0.14 (0.12–0.17)	<0.0001	0 (0.0%)	767 (26.5%)	0.0006 (0.00004–0.01)	<0.0001
LGI-1	<i>Listeria</i> genomic island, BC & desiccation resistance ^{48,49}	4 (0.1%)	0 (0.0%)	4 (0.1%)	4.76 (0.26–100.00)	0.29	2 (0.1%)	2 (0.1%)	0.83 (0.12–5.88)	0.86	0 (0.0%)	4 (0.1%)	0.13 (0.01–2.44)	0.17
LGI-2	<i>Listeria</i> genomic island, Cd & As resistance ⁵⁰	346 (6.5%)	10 (8.4%)	336 (6.4%)	1.33 (0.69–2.56)	0.41	208 (7.2%)	138 (5.7%)	1.28 (1.02–1.59)	0.03	211 (8.7%)	135 (4.7%)	1.92 (1.56–2.44)	<0.0001
LGI-3	<i>Listeria</i> genomic island, Cd & As resistance ⁵¹	171 (3.2%)	0 (0.0%)	171 (3.3%)	0.12 (0.01–2.00)	0.14	113 (3.9%)	58 (2.4%)	1.67 (1.19–2.27)	0.00	106 (4.3%)	65 (2.2%)	2.00 (1.45–2.70)	<0.0001

The Null hypothesis (H0) for all tests stated the odds ratio is equal to 1. Conversely, the alternative hypothesis (H1) suggested the odds ratio is different from 1. Abbreviations: AR, antibiotic resistance; CI, confidence interval; As, arsenic; BC, benzalkonium chloride; Cd, cadmium. Chi-2 tests were employed to estimate the significance of the odds ratio. ^aIsolates with acquired antibiotic resistance (AR) versus those without acquired AR. ^bClinical isolates versus food isolates. ^cLm lineage I isolates versus lineage II isolates.

Table 3: Prevalence of other acquired resistance traits detected in the *Listeria monocytogenes* isolates used in this study.

unfavorable outcome in meningitis patients, despite antimicrobial therapy,⁵² suggesting a possible role of biocide resistance efflux pumps in antimicrobial resistance. In addition, exposure to benzalkonium chloride has been shown to increase resistance to ciprofloxacin.⁵³ Taken together, these results highlight the importance of monitoring also biocide resistance not only to prevent persisting *Lm* environment contamination but also to prevent the emergence of cross-resistance to antimicrobials. Interestingly, we observed that acquired antimicrobial resistance genes were significantly more prevalent in isolates carrying *bcrABC* and *emrC* genes encoding resistance to benzalkonium chloride disinfectants and/or SSI-1 stress islands ($p < 10^{-4}$, Fisher's exact test) and that these resistant genes were significantly more prevalent and diverse in food and in lineage II isolates (Table 3), highlighting a potential role of the production environments in the co-selection of resistance traits.

Nevertheless, resistance to antimicrobials used in patients with listeriosis remains rare. General breakpoints based on antimicrobials pharmacokinetics and pharmacodynamics are appropriate for antimicrobials commonly used for the treatment of listeriosis.

While our findings may be affected by geographical variations, temporal changes, sampling biases and/or strain diversity, the low prevalence of acquired resistance is in line with earlier studies in France and in large-scale studies in other world regions.^{26,28,54} Alarming results on resistance to first-line aminopenicillins (such as amoxicillin, penicillin and ampicillin) reported in a few countries, sometimes without clearly mention of the methodology or breakpoints used (for a review see Rostamian and colleagues),⁵⁵ were not found in this study. Thus, those should be considered with caution⁵ and authors should be encouraged to confirm results on atypical resistances with interlaboratory studies and genome sequencing prior publication. At a time of fast-paced spreading of resistance or reduced susceptibility towards first-line antimicrobials among human pathogens, these data provide reassuring information for clinicians in charge of patients with invasive listeriosis.

In conclusion, no increased resistance of *Lm* to antimicrobials was observed in France and overseas territories during the 8-year period, and reference treatment options should therefore be considered as microbiologically effective for the treatment of listeriosis. Acquired antimicrobial resistance in *Lm* could be predicted from genomes with high accuracy. A higher prevalence of acquired antimicrobial resistance genes was observed in food isolates (3.74%), predominantly belonging to lineage II, as compared to clinical isolates (0.98%, for the same period), which were predominantly from lineage I, and in isolates harbouring disinfectants or stress resistance traits (specifically *bcrABC*, *emrC* and SSI-1), suggesting co-selection of resistance in food-production environments. Continuous genomic surveillance of antimicrobial resistance, both within and outside the

clinical setting, is critical to detect emerging patterns of *Lm* resistance.

Contributors

ML initiated the project. Project coordination: ML, AM, AL. Data collection: AL, CC. Isolates selection: AL. Isolates sequencing and antimicrobial susceptibility testing: GV, NTR, HBD, PT. Sequence analysis: AM. Phenotypic analysis: AM and AL. Statistical analyses: YM, AM. AM wrote the manuscript based on a first draft from AL, with contributions and comments from ML, CC and YM.

Data sharing statement

All sequences of antimicrobial resistant isolates are publicly available at the European Nucleotide Archive (BioProject PRJEB12738). Profiles of the remaining isolates are available in <https://bigsd.b.pasteur.fr/listeria>.

Declaration of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.lanep.2023.100800>.

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