Spread of carbapenemase-producing *Morganella* spp from 2013 to 2021: a comparative genomic study

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Summary

Background *Morganella* spp are opportunistic pathogens involved in various infections. Intrinsic resistance to multiple antibiotics (including colistin) combined with the emergence of carbapenemase producers reduces the number of active antimicrobials. The aim of this study was to characterise genetic features related to the spread of carbapenem-resistant *Morganella* spp.

Methods This comparative genomic study included extensively drug-resistant *Morganella* spp isolates collected between Jan 1, 2013, and March 1, 2021, by the French National Reference Center (NRC; n=68) and European antimicrobial resistance reference centres in seven European countries (n=104), as well as one isolate from Canada, two reference strains from the Pasteur Institute collection (Paris, France), and two colistin-susceptible isolates from Bicêtre Hospital (Kremlin-Bicêtre, France). The isolates were characterised by whole-genome sequencing, antimicrobial susceptibility testing, and biochemical tests. Complete genomes from GenBank (n=103) were also included for genomic analysis, including phylogeny and determination of core genomes and resistomes. Genetic distance between different species or subspecies was performed using average nucleotide identity (ANI). Intrinsic resistance mechanisms to polymyxins were investigated by combining genetic analysis with mass spectrometry on lipid A.

Findings Distance analysis by ANI of 275 isolates identified three groups: *Morganella psychrotolerans*, *Morganella morganii* subspecies *sibonii*, and *M morganii* subspecies *morganii*, and a core genome maximum likelihood phylogenetic tree showed that the *M morganii* isolates can be separated into four subpopulations. On the basis of these findings and of phenotypic divergences between isolates, we propose a modified taxonomy for the *Morganella* genus including four species, *Morganella psychrotolerans*, *Morganella sibonii*, *Morganella morganii*, and a new species represented by a unique environmental isolate. We propose that *M morganii* include two subspecies: *M morganii* subspecies *morganii* (the most prevalent) and *M morganii* subspecies *intermedius*. This modified taxonomy was supported by a difference in intrinsic resistance to tetracycline and conservation of metabolic pathways such as trehalose assimilation, both only present in *M sibonii*. Carbapenemase producers were mostly identified among five high-risk clones of *M morganii* subspecies *morganii*. The most prevalent carbapenemase corresponded to NDM-1, followed by KPC-2, and OXA-48. A cefepime–zidebactam combination was the most potent antimicrobial against the 172 extensively drug-resistant *Morganella* spp isolates in our collection from different European countries, which includes metallo-β-lactamase producers. Lipid A analysis showed that the intrinsic resistance to colistin was associated with the presence of L-ARA4N on lipid A.

Interpretation This global characterisation of, to our knowledge, the widest collection of extensively drug-resistant *Morganella* spp highlights the need to clarify the taxonomy and decipher intrinsic resistance mechanisms, and paves the way for further genomic comparisons.

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Introduction

Morganella morganii is a facultative, anaerobic, Gram-negative rod belonging to the order Enterobacterales. Initially reported as *Proteus morganii* in 1907, it was reclassified as *Morganella morganii* genus novum in 1943.[1] Currently, the *Morganella* genus is composed of two species: *M morganii* and *Morganella psychrotolerans*.[2] Whereas *M morganii* is frequently encountered in clinical specimens, the psychrotolerant *M psychrotolerans* is associated with seafood poisoning by production of histamine.[3] In 1992, biochemical analysis was used to divide *M morganii* into two subspecies on the basis of their ability to use of trehalose,[4] positive for *M morganii* subspecies *sibonii* and negative for *M morganii* subspecies *morganii*.

M morganii is an opportunistic pathogen responsible for a wide variety of infections, such as urinary tract infections,

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Research in context

Evidence before this study

On Oct 5, 2023, we searched all published reports available in PubMed using the terms "*Morganella*" and "carbapenemase" with no language restrictions and no publication date restrictions. We identified 55 articles, 18 of which reported single isolates of carbapenemase-producing *Morganella morganii*, 23 did not directly study the *Morganella* genus, and 14 described evaluation of the efficiency of novel antibiotics or detection tests. Only one study aimed to assess the antimicrobial susceptibility of a global collection of *Proteus*, *Providencia,* and *Morganella* isolated from patients admitted to hospital with intra-abdominal and urinary tract infections. However, this collection included only seven *M morganii* isolates. On March 1, 2021, the cutoff for inclusion in our study, only 104 assembled genomes of *Morganella* spp were available in the GenBank database. One study reported 40 multidrug-resistant *M morganii* isolates recovered from three hospitals in China from 2014 to 2020. This study included only two carbapenemase-producing *M morganii* isolates (one OXA-48 and one IMP-1). A report of KPC-producing *M morganii* in Japan and a longitudinal study of carbapenemase-producing Enterobacterales in Taiwan did not focus on *Morganella*. We also searched PubMed on Oct 5, 2023, for the terms '*Morganella sibonii*" or "*Morganella psychrotolerans*" in all published reports with no language restrictions and no publication date restrictions. Our search identified 21 articles, none of which provided a detailed characterisation of *Morganella* spp, including antimicrobial resistance and phylogenetic grouping, on the basis of genomic data.

Added value of this study

To our knowledge, this global characterisation involved the widest collection of multidrug-resistant *Morganella* spp isolates ever reported. In addition, 145 of 172 multidrug-resistant *Morganella* isolates included in our collection were carbapenemase producers, for which therapeutic alternatives are scarce due to intrinsic resistance to last-resort molecules, such as polymyxin. We found that cefepime–zidebactam and ceftazidime–avibactam were the most potent antimicrobials against extensively drug-resistant *Morganella* spp isolates, except for metallo-β-lactamase (MBL) producers. In MBL-producing isolates, the aztreonam–avibactam combination was the most efficient. We also observed differences between *Morganella* species that suggested a need to change the genus taxonomy. We propose the *Morganella* genus should be divided into four species, *Morganella psychrotolerans*, *Morganella sibonii*, *Morganella morganii,* and a new species represented by a unique strain. According to our proposed taxonomy, *M morganii* includes two subspecies: *M morganii* subspecies *morganii* (the most prevalent) and *M morganii* subspecies *intermedius*. We showed that this refined taxonomy correlated with intrinsic resistance to tetracycline, which was found only in *M sibonii*, as well as several metabolic pathways (eg, trehalose assimilation and type VI secretion system). In addition, we highlighted high-risk clones of carbapenemase-producing *M morganii* subspecies *morganii* that have already disseminated worldwide. Combining whole-genome sequencing data with epidemiological investigations, we showed that a cutoff of 20 single nucleotide polymorphisms could be used to discriminate clonally related from sporadic independent isolates. The intrinsic resistance of *Morganella* spp to polymyxins was well known but the underlying mechanism remained unclear. We showed that the addition of L-ARA4N on lipid A of *Morganella* isolates is involved in this process.

Implications of all the available evidence

The identification of high-risk clones among extensively drug-resistant *Morganella* spp paves the way for future investigations to better understand and hopefully limit the spread of these bacteria. Additionally, our results identified new components and virulence factors of some *Morganella* species (eg, type VI secretion system in *M sibonii*) that deserve further investigation since they might be implicated in the bacterial pathogenesis (eg, different infection types) of this genus.

septic shock, surgical site infections, osteomyelitis, and pneumonia.[5,6] Moreover, *M Morganii* is increasingly prevalent worldwide and represented 99 (1·27%) of 7800 nosocomial infections observed in a national study in France in 2017.[7] Except for a cluster of *M morganii* infections reported in the late 1970s,[8] no other outbreak has been studied at the microbial and genomic levels. Accordingly, the molecular epidemiology of *Morganella* spp recovered from clinical samples has never been explored.

Morganella spp isolates are intrinsically resistant to colistin, macrolides, fosfomycin, amoxicillin, and first and second generation cephalosporins (due to the production of a class C β-lactamase, $bla_{\text{DHA-like}}$), and have reduced susceptibility to imipenem due to the low affinity of their penicillin binding protein, PBP-2.[6] The treatment of infections caused by Enterobacterales (including *Morganella*) often involves β-lactams, including carbapenems. Carbapenem resistance in Enterobacterales is due to the production of extended spectrum β-lactamases (ESBLs) or overproduced AmpC β-lactamases associated with decreased outer-membrane permeability or to the production of carbapenemases, enzymes with substantial hydrolytic activity towards carbapenems.[9] The most prevalent carbapenemases are Ambler's class A enzymes (mainly KPC-like enzymes), the Ambler's class B metallo-β-lactamases (MBLs; including NDM-type, VIM-type, and IMP-type), and Ambler's class D carbapenem-hydrolysing β-lactamases of OXA-48-like.[10] Carbapenemase-producing *M morganii* are rarely reported. One study reported 40 multidrug resistant *M morganii* isolates recovered from three hospitals in China from 2014 to 2020.[11] However, the most prevalent carbapenemases produced by *M morganii* are NDM-like enzymes.[12,13] More sporadically, OXA-48-like and KPC-like enzymes have also been reported in

M morganii.[14,15] To our knowledge, only one report of GES-5 carbapenemase in *M morganii* has been published.[16]

The aim of this study was to clarify the genetic diversity of carbapenemase-producing *Morganella* spp spreading in France and in Europe more generally, to decipher antibiotic resistance mechanisms, and to better characterise the taxonomy of this genus.

Methods

Study design and strain collection

This was a large-scale epidemiological study in which, all *Morganella morganii* isolates with a reduced susceptibility profile to ertapenem or meropenem (regardless of imipenem susceptibility) sent to the French National Reference Center (NRC) for Antimicrobial Resistance (Kremlin-Bicêtre, France) from Jan 1, 2013, to March 1, 2021, were included. The bacterial isolates referred to the NRC were recovered from clinical and screening human specimens collected in French microbiology laboratories. 104 *M morganii* isolates (also collected from Jan 1, 2013, to March 1, 2021) from Germany (n=32), Belgium (n=26), England (n=17), Austria (n=3), the Netherlands (n=4), Poland (n=1), and Czech Republic (n=21) suspected to produce a carbapenemase (according to European Committee on Antimicrobial Susceptibility Testing [EUCAST] guidelines for the detection of resistance mechanisms) and referred to European antimicrobial resistance reference centres were also included. Antimicrobial resistance reference centres are part of the European Antimicrobial Resistance Genes Surveillance Network, allowing the sharing of isolates for research purposes. European centres that shared isolates for this study also provided some epidemiological information linked to the isolates (ie, date of isolation and whether each isolate was suspected to be part of an outbreak). One additional carbapenemase-producing *M morganii* isolate from Canada was used in this study (provided by Andrew Walky, University of Manitoba, Winnipeg, MB, Canada).[17] Two *M morganii* subspecies *sibonii* reference strains (CIP 103648 and CIP 103649) were provided by the Pasteur Institute collection (Pasteur's Institute, Paris, France), and two *M morganii* isolates presenting a wild-type phenotype (O86D10 and O86E1) from Bicêtre Hospital (Kremlin-Bicêtre, France) were used as recipient strains for plasmid transformation (appendix pp 5, 22–33).

This was a retrospective study investigating carbapenem-resistant bacteria. This study does not include patient data, therefore no ethics committee was necessary for this study.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all isolates from the French NRC, European countries, and the two wild-type isolates by the disc diffusion method on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to EUCAST guidelines as updated in 2021.[18] Minimum inhibitory concentrations (MICs) of temocillin, cefepime–zidebactam, ceftazidime, ceftazidime–avibactam, ceftolozane–tazobactam, ertapenem, imipenem, imipenem–relebactam, meropenem, meropenem–vaborbactam, aztreonam, aztreonam–avibactam, and colistin (polymyxin E) were determined by broth microdilution (Sensititre, Thermofisher, Dardilly, France). Carbapenemase detection was performed using the Carba NP test as previously described,[19] followed by immunochromatographic detection of the carbapenemase enzyme using the NG-Carba5 test (NG Biotech, Guipry, France). The MIC of tetracycline was measured using the E-test (Biomérieux, La Balme les Grottes, France). MICs of eravacycline and tigecycline were measured using broth microdilution (Sensititre, Thermofisher, Dardilly, France).

Whole-genome sequencing

All *Morganella* spp from the French NRC, the European antimicrobial resistance reference centres, the Canadian isolate, the two isolates from Pasteur's Institute collection, and two from Bicêtre Hospital were sequenced using NextSeq500 technology (Illumina, Evry-Courcouronnes, France) as previously described (appendix p 2).[20] De novo assembly and read mappings were performed using CLC Genomics Workbench version 12.0 (Qiagen, Les Ulis, France). Long-read sequencing was performed on *M morganii* 24A3 using PacBio technologies (Macrogen, Amsterdam, Netherlands) as described previously.[20]

Dataset of *Morganella* spp genomes and phylogeny analysis

A dataset of *Morganella* spp genomes from our isolate collection (NRC, Europe [not France], Pasteur Institute, and Bicêtre Hospital) and from GenBank (all available whole-genome sequences) was used for bioinformatic analysis. We estimated the similarity between genomes by calculating pairwise genetic distances with Mash (version 2.1.1) and FastANI (version 1.33). The Mash genetic distance (D) correlates with average nucleotide identity (ANI): $D \approx 1 - (ANI/100)$.

For **GenBank** see https://www.ncbi.nlm.nih.gov/genbank/

For **Mash** see https://github.com/marbl/Mash/releases/tag/v2.1.1

For **FastANI** see https://github.com/ParBLiSS/FastANI/releases/tag/v1.33

Bioinformatic analyses

The acquired antimicrobial resistance genes were identified in all genomes (ie, from our isolate collection and GenBank) using Resfinder server (version 3.1) with default parameters. Sequence alignments were performed using ClustalW. The plasmids of clinical isolates were analysed by searching for replicase genes using PlasmidFinder (version 2.1) with default parameters and by a manual search for genes showing homology using BLAST in the GenBank database with a replicase gene. Transposons were named according to the Transposon Registry database.[21]

For **Resfinder** see https://cge.cbs.dtu.dk/services/ResFinder/ See Online for appendix

For **ClustalW** see https://www.genome.jp/tools-bin/clustalw

For **PlasmidFinder** see https://cge.food.dtu.dk/services/PlasmidFinder/

For **BLAST** see https://blast.ncbi.nlm.nih.gov/Blast.cgi

Identification of pangenomes and core genomes

For the identification of core and pangenomes of all *Morganella* spp except *M psychrotolerans*, we used the PanACoTA (version 1.2.06) pipeline with default parameters.[22] We applied the quality control module by applying the following criteria as parameters of the PanACoTA tool:

max_L90=100 (where max_L90 is the maximum allowed count of smallest number of contigs whose length sum makes up 90% of genome size) and max_contigs=999 (where max_contigs is the maximum number of contigs allowed). We reran the annotation inside the pipeline on the remaining *M morganii* subspecies *morganii* and *M morganii* subspecies *sibonii* genomes using Prokka for homogeneousness. The pangenome was built in the pipeline (MMseqs2 version 13-45111 dependency) with identity parameters set at 80%, resulting in 22 952 families of homologous proteins. Core genomes were inferred from the pangenome with a tolerance parameter of 95% (a gene is core if its presence is >95%). Three core genomes were computed: the first on the whole set, the second on the subset comprising *M morganii* subspecies *morganii*, and the third on the subset comprising *M morganii* subspecies *sibonii*.

For **Tracer** see https://github.com/beast-dev/tracer/releases/tag/v1.7.2

For **FigTree** see http://tree.bio.ed.ac.uk/software/figtree/

For **Prokka** see https://github.com/tseemann/prokka

For **MMseqs2** see https://github.com/soedinglab/MMseqs2/releases/tag/13-45111

Single nucleotide polymorphism-based and core-genome phylogeny

The genomes were annotated using the RAST server. Single nucleotide polymorphisms (SNPs) were identified on the whole genomes using CSIphylogeny (version 1.4) with the following parameters: minimum depth at SNP position set at 10X, minimum distance between SNPs set at 10 bp, minimum SNP quality set at 30 SNPs. The phylogeny was then inferred using the CSIphylogeny server and visualised using iTOL software (version 4.11). Phylogenetic analysis was performed on isolates from the French NRC as well as all *Morganella* spp isolates. Where epidemiological data linked to individual isolates were available (from France and other European countries), we took them into account in our interpretation of phylogenetic clustering. Phylogenetic analysis of specific clones was also performed.

For **RAST** see https://rast.nmpdr.org/

For **CSIphylogeny** see https://cge.food.dtu.dk/services/CSIPhylogeny/

For **iTOL** see itol.embl.de/

Core genome phylogeny was performed using the genomes of *M morganii sensu lato* and of *M psychrotolerans* (used here to assess the genetic distance of this species from *M morganii*) to confirm results obtained using whole-genome phylogeny. Trees were generated using core genomes obtained with PanACoTA with multiple alignments with MAFFT and 12 phylogenetic trees obtained using IQ-TREE (version 2.0.613) with the TEST model option. A general time reversible model with unequal rates and unequal base frequencies plus empirical base frequencies was selected according to Bayesian information criterion.

For **MAFFT** see https://mafft.cbrc.jp/

For **IQ-TREE** see http://www.iqtree.org/

Temporal signal

The strength of the temporal signal of the molecular phylogeny was evaluated by root-to-tip linear regression on the core-genome phylogenetic tree of clone I using TempEST (version 1.5.2) with standard parameters.[23] We then used the resulting alignment in a Bayesian analysis using BEAST (version 1.10.4; run with a Markov chain Monte Carlo length of 1×10^9, sampling every 5×10^3 steps).[24] The model with the best fit was a generalised time reversible substitution model, log-normal relaxed clock and coalescent constant population and was chosen using IQ-TREE with standard parameters. Time estimation was performed using Tracer (version 1.7.2) with default parameters and TreeAnnotator (version 2.6.0), and finally visualised using FigTree (version 1.4.4).

Assessing trehalose assimilation and intrinsic tetracycline resistance in *M sibonii*

M sibonii has been shown to differ from *M morganii* subspecies *morganii* by assimilation of trehalose and we aimed to confirm this difference by cloning a trehalose operon from *M sibonii* GER-11 to *M morganii* subspecies *morganii* O86D10 and analysing the trehalose operon.[25] Whole-cell DNA was extracted using the Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Ozyme, Saint-Quentin, France) according to the manufacturer's instructions as previously described.[26] DNA from *M sibonii* GER-11 was used as a template for the amplification of the trehalose operon (7517 bp) followed by cloning and expression in electrocompetent *M morganii* subspecies *morganii* O86D10 (see appendix pp 2–3 for detailed procedure). Biochemical characterisation, including trehalose assimilation of the collection, was performed using Api20E and Api50CH systems (BioMérieux, La Balme les Grottes, France) according to the manufacturer's recommendations. We also evaluated the functionality of the *tetD* gene, which is responsible for natural resistance to tetracycline in *M sibonii*, by cloning and expressing it in *Escherichia coli* (see appendix pp 2–3 for detailed procedure).

MALDIxin test

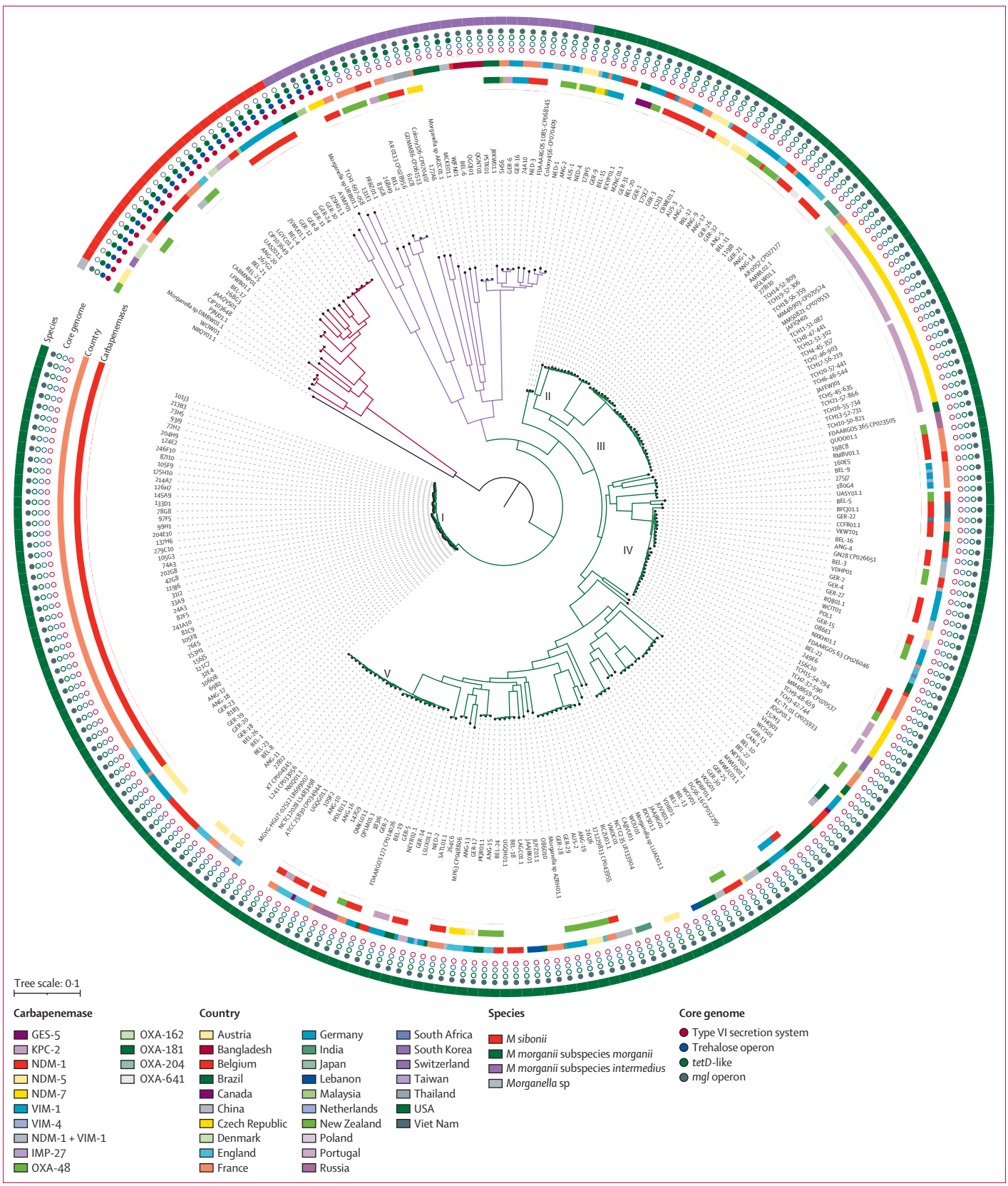
Morganella spp are known to be naturally highly resistant to polymyxin. Although the underlying mechanism remains unknown, modifications of the lipid A region of the lipopolysaccharide are thought to be involved, such as 4-amino-4-deoxy-L-arabinose (L-ARA4N) binding. We sought to detect lipid A modifications involved in colistin–polymyxin resistance by using the MALDIxin test as previously described.[27] We applied the MALDIxin test to ten colistin-resistant isolates (MIC >256 mg/L) and two susceptible isolates (BEL-5 and BEL-6) from our collection. Colistin-resistant isolates were selected to be representative of the genetic diversity of the collection (ie, belonging to different clones).

Role of the funding source

There was no funding source for this study.

Results

During the study period, 68 non-duplicate carbapenemase-producing *M morganii* isolates were collected at the French NRC, representing 0·46% of the 14 672 carbapenemase-producing Enterobacterales isolates collected in total. These *M morganii* isolates were recovered from eight French regions (appendix p 5), and 43 (63%) were recovered from the same area in southwest France (appendix p 5). Phylogenetic analysis performed on all 68 *M morganii* isolates showed that among the 53 NDM-1-producing *M morganii*, 41 (77%) belonged to a single clone (clone I; appendix p 2).


Species
Core genome
Country
Carbapenemases
I
II
III
IV
V
Tree scale: 0·1
Carbapenemase
GES-5
KPC-2
NDM-1
NDM-5
NDM-7
VIM-1
VIM-4
NDM-1 + VIM-1
IMP-27
OXA-48
OXA-162
OXA-181
OXA-204
OXA-641
Country
Austria
Bangladesh
Belgium
Brazil
Canada
China
Czech Republic
Denmark
England
France
Germany
India
Japan
Lebanon
Malaysia
Netherlands
New Zealand
Poland
Portugal
Russia
South Africa
South Korea
Switzerland
Taiwan
Thailand
USA
Viet Nam
Species
M sibonii
M morganii subspecies morganii
M morganii subspecies intermedius
Morganella sp
Core genome
Type VI secretion system
Trehalose operon
tetD-like
mgl operon

This clone included 39 isolates collected in three cities located 22 km, 72 km, and 94 km from each other in southwest France (suggesting patient-to-patient cross-transmission; we consider these isolates as probably part of an outbreak, which henceforth we refer to as the French outbreak), and two isolates collected from a distant area in northeast France (appendix p 5).

The analysis of antibiotic resistance gene content in the French outbreak isolates showed that the main clone identified in southwest France carried the bla_{NDM-1} carbapenemase-encoding gene, the $bla_{CTX-M-15}$ ESBL gene, and two copies of the $bla_{DHA-like}$ cephalosporinase gene. In addition, clone I encoded two 16S RNA methylases, ArmA and RmtC, and two aminoglycoside-modifying enzymes, AAC(6′)-Ib and AadA1, conferring resistance to all aminoglycosides tested (MIC >32 mg/L for tobramycin, netilmicin, gentamicin, and amikacin). Resistance to quinolones (MIC >32 mg/L for ciprofloxacin and levofloxacin) was mediated by known mutations in GyrA (Ser83Ile) and ParC (Ser84Ile), and additional mechanisms such as the production of QnrA1, a protein that protects DNA gyrase and topoisomerase IV, and AAC(6′)-Ib-cr, an aminoglycoside acetyltransferase able to modify ciprofloxacin. Combined with the intrinsic resistance to polymyxins and tigecycline, acquired resistance determinants were responsible for full resistance to all tested molecules (appendix pp 38–40). Attempts to transfer the bla_{NDM-1} gene failed (appendix p 3), suggesting a chromosome location of the bla_{NDM-1} gene, which was confirmed by PacBio-based long-read sequencing inside novel transposon Tn*7340* (appendix pp 6–7).

To determine if the main clonal group (comprising clone I) observed in France had already spread abroad, we analysed the genome sequences of additional 104 *Morganella* spp isolates with decreased susceptibility to ertapenem or meropenem collected from seven reference centres across Europe and of 103 *Morganella* spp from GenBank (appendix pp 23–32). Resistome analysis identified a wide variety of carbapenemases produced by these isolates (figure 1 and table). Of note, a wide diversity of carbapenemases was identified in Germany, including NDM-1, NDM-5, VIM-1, OXA-48, OXA-181, and OXA-641 (a variant of OXA-372 reported only once in *Citrobacter freundii*). By contrast, in the Czech Republic, KPC-2 carbapenemase was highly prevalent (in 16 [76%] of 21 isolates).

Distance analysis was conducted by creating an ANI and a Mash distance similarity matrix including the genomes of 270 *M morganii* isolates (104 from Europe [outside of France], one from Canada, two from the Pasteur's Institute collection, two from Bicêtre Hospital, 68 from the NRC, and 93 from the GenBank database) and five genomes of

Figure 1: **Single nucleotide polymorphism-based phylogenetic analysis of the 270 isolates of *Morganella* spp**
The phylogenetic tree was constructed using CSIphylogeny version 1.4 and visualised using iTOL version 4.11. Species and subspecies are defined according to our proposed modified taxonomy of the *Morganella* genus. Produced carbapenemases are indicated by coloured squares. I–V are the five main clones of *M morganii* subspecies *morganii*.

	M morganii	*M sibonii*	*M psychrotolerans*
Number of genomes	247	23	5
Mean genome size, Mbp	3·97 (0·19)	4·14 (0·22)	4·21 (0·09)
Mean contig number	166·21 (286·14)	154·95 (188·31)	40·40 (15·01)
N50, kbp	597·97 (1195·86)	308·89 (852·37)	386·40 (170·59)
β-lactam resistance			
Class A carbapenemases			
bla_{KPC-2}	26 (11%)	0	0
bla_{GES-5}	1 (<1%)	0	0
Class B carbapenemases			
bla_{NDM-1}	75 (30%)	6 (26%)	
bla_{NDM-5}	10 (4%)	0	0
bla_{NDM-7}	3 (1%)	0	0
bla_{VIM-1}	3 (1%)	1 (4%)	0
$bla_{VIM-1-like}$	1 (<1%)	0	0
bla_{VIM-4}	2 (1%)	0	0
bla_{IMP-27}	1 (<1%)	0	0
Class D carbapenamases			
bla_{OXA-48}	22 (9%)	5 (22%)	0
$bla_{OXA-162}$	1 (<1%)	0	0
$bla_{OXA-181}$	2 (1%)	0	0
$bla_{OXA-204}$	1 (<1%)	0	0
$bla_{OXA-641}$	1 (<1%)	0	0
ESBLs or cephalosporinases			
$bla_{DHA/MOR}$-like	247 (100%)	23 (100%)	0
$bla_{CTX-M-1}$	0	1 (4%)	0
$bla_{CTX-M-3}$	1 (<1%)	0	0
$bla_{CTX-M-14}$	4 (2%)	0	0
$bla_{CTX-M-15}$	1 (<1%)	0	0
bla_{CMY-2}	2 (1%)	0	0
bla_{CMY-4}-like	6 (2%)	0	0
bla_{CMY-16}	0	1 (4%)	0
bla_{CMY-16}-like	2 (1%)	0	0
bla_{SHV-12}	6 (2%)	0	0
bla_{TEM-2}	1 (<1%)	0	0
bla_{VEB-6}-like	1 (<1%)	0	0
bla_{OXA-35}	1 (<1%)	0	0
Penicillinases			
bla_{TEM-1}-like	43 (14%)	0	0
$bla_{TEM-110}$-like	2 (1%)	0	0
bla_{CARB-2}-like	10 (4%)	0	0
bla_{OXA-1}	30 (12%)	0	0
bla_{OXA-9}	2 (1%)	0	0
bla_{OXA-10}-like	5 (2%)	0	0
bla_{OXA-10}	0	1 (4%)	0
Non-β-lactam resistance			
Aminoglycosides			
aadA1-like	109 (44%)	6 (26%)	0
aadA2-like	39 (16%)	1 (4%)	0
aadA5	19 (8%)	1 (4%)	0
aadA7	1 (<1%)	0	0
aadA12-like	6 (2%)	0	0

(Table continues on next page)

	M morganii	*M sibonii*	*M psychrotolerans*
(Continued from previous page)			
aadA13-like	2 (1%)	0	0
aadA16-like	2 (1%)	0	0
aadA17-like	1 (<1%)	0	0
aadA24-like	9 (4%)	1 (4%)	0
aadB-like	13 (5%)	6 (26%)	0
strA-like	37 (15%)	6 (26%)	0
strB-like	36 (15%)	6 (26%)	0
aac(3′)-II-like	44 (18%)	0	0
aac(3′)-IVa-like	6 (2%)	2 (9%)	0
aac(6′)Ib-like	116 (47%)	1 (4%)	0
aac(6′)-aph(2″)-like	1 (<1%)	0	0
aph(3′)-I-like	36 (15%)	0	0
aph(3′)-VIa-like	16 (6%)	1 (4%)	0
aph(4)-Ia	6 (2%)	1 (4%)	0
armA-like	42 (17%)	0	0
rmtB-like	4 (2%)	0	0
rmtC-like	39 (16%)	0	0
aph(3′)-Ic	0	2 (9%)	0
Fluoroquinolones			
qnrA1-like	43 (17%)	0	0
qnrB1-like	2 (1%)	0	0
qnrD	0	10 (43%)	0
qnrB2	1 (<1%)	0	0
qnrB6	1 (<1%)	0	0
qnrB19	1 (<1%)	0	0
qnrB32-like	6 (2%)	0	0
qnrB58-like	1 (<1%)	0	0
qnrB66-like	1 (<1%)	0	0
qnrD-like	17 (7%)	0	0
qnrS1-like	5 (2%)	0	0
qnrS2-like	1 (<1%)	0	0
qepA-like	2 (1%)	0	0
Chloramphenicol			
catA1-like	63 (26%)	0	0
catA2-like	111 (45%)	16 (70%)	0
catB2-like	8 (3%)	1 (4%)	0
catB3-like	71 (29%)	3 (13%)	0
FlorR-like	14 (6%)	4 (17%)	0
cmlA-like	7 (3%)	0	0
Tetracycline			
tetA-like	23 (9%)	1 (4%)	0
tetB-like	124 (50%)	1 (4%)	0
tetD-like	3 (1%)	23 (100%)	0
tetL-like	1 (<1%)	0	0
tetY-like	1 (<1%)	1 (4%)	0
Macrolides			
mph(A)-like	41 (17%)	0	0
mph(E)-like	52 (35%)	0	0
ere(A)-like	1 (<1%)	0	0
ere(B)-like	10 (4%)	0	0
erm(42)-like	4 (2%)	0	0
erm(B)-like	9 (4%)	0	0
msr(E)-like	52 (21%)	0	0
mph(A)	0	2 (9%)	0
Colistin			
mcr-1	2 (1%)	0	0
Rifampicin			
arr-2	2 (1%)	0	0
arr-3	37 (15%)	0	0
Trimethoprim			
dfrA1-like	103 (42%)	0	0
dfrA7	1 (<1%)	0	0
dfrA12-like	14 (6%)	0	0
dfrA14-like	15 (6%)	6 (26%)	0
dfrA15	4 (2%)	0	0
dfrA17-like	19 (8%)	0	0
dfrA18-like	9 (4%)	0	0
dfrA24-like	1 (<1%)	0	0
dfrA27-like	2 (1%)	0	0
dfrA30-like	1 (<1%)	0	0
dfrA1	0	7 (30%)	0
dfrA12	0	1 (4%)	0
dfrA16-like	0	3 (13%)	0
dfrA17	0	1 (4%)	0
Sulfamides			
sul1-like	144 (58%)	0	0
sul2-like	46 (19%)	0	0
sul3	3 (1%)	0	0
sul1	0	3 (13%)	0
sul2	0	8 (36%)	0

Data are mean (SD) or n (%). Species are defined according to our proposed modified taxonomy of the *Morganella* genus. ESBL=extended-spectrum β-lactam.

Table: **Genetic features and diversity of acquired resistance genes of *Morganella* spp isolates**

M psychrotolerans from the GenBank database (appendix pp 9–10). Five sequences from the GenBank collection were not included in this analysis because they did not pass the quality control step. Three groups were identified: *M psychrotolerans* (five genomes), *M morganii* subspecies *sibonii* (23 genomes), and *M morganii* subspecies *morganii* (247 genomes). *M psychrotolerans* was found to be very distant from *M morganii* (less than 86% ANI; appendix pp 9–10). A core genome maximum likelihood phylogenetic tree confirmed this result (appendix p 11); hence, due to the large genetic distance of *M psychrotolerans* from *M morganii*, only genomes classified as *M morganii* were kept for further analysis, corresponding to 270 genomes. The core genome maximum likelihood phylogenetic tree also showed that the *Morganella morganii* isolates can be separated into four subpopulations (figure 1, appendix p 11).

The first subpopulation corresponded to what was formerly *M morganii* subspecies *sibonii* and included 23 isolates with approximately 92% identity with the *M morganii* subspecies *morganii* and less than 85% with *M psychrotolerans*.

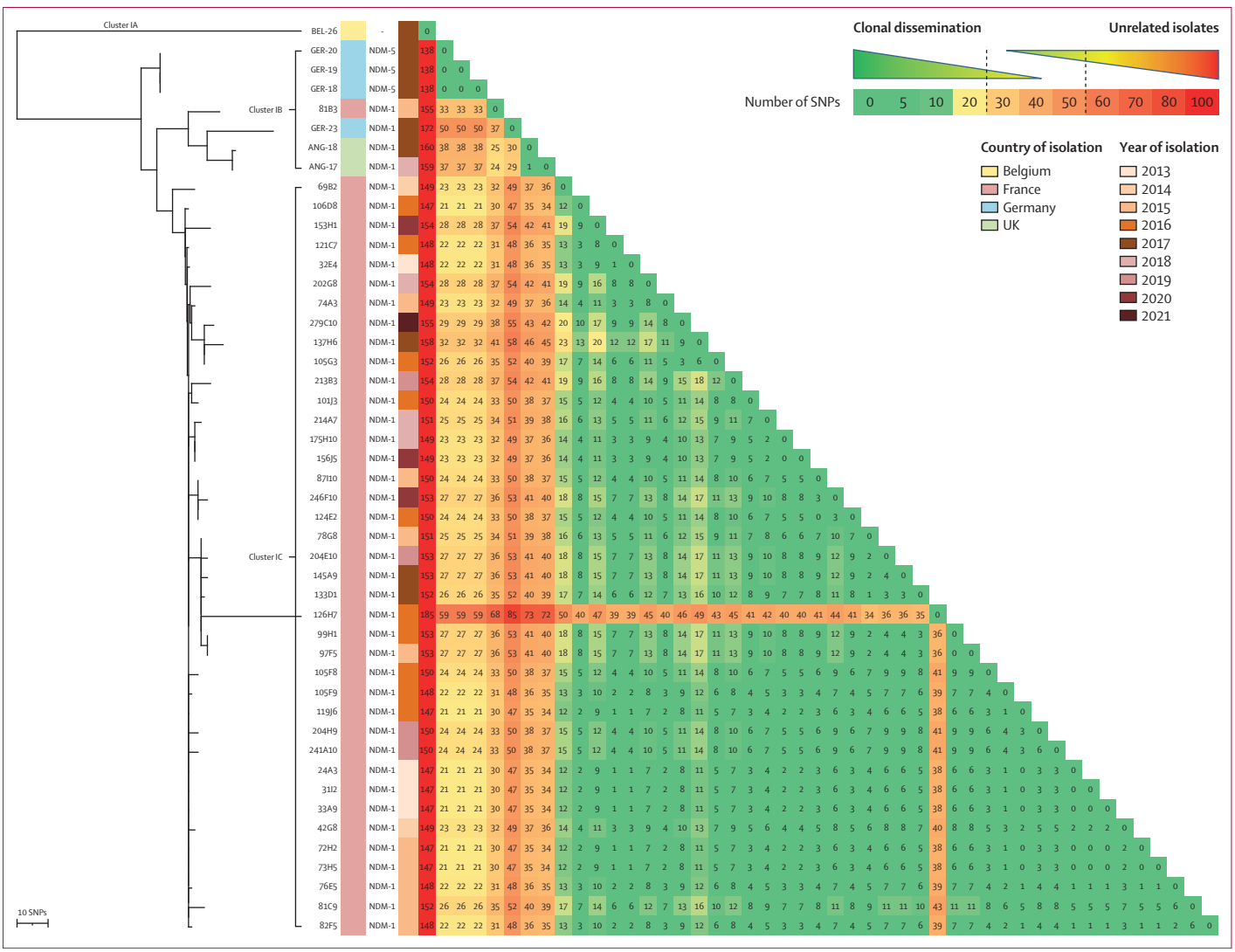


Figure 2: **Analysis of SNPs in *Morganella morganii* subspecies *morganii* cluster I**

This SNP-based phylogenetic tree was obtained by comparing all isolates from *M morganii* subspecies *morganii* cluster I. An SNP-based matrix obtained from CSIphylogeny version 1.4 was visualised. Year of isolation, country, and carbapenemase of each isolate is indicated next to the isolate name. SNP=single nucleotide polymorphism.

We proposed that this subpopulation be reclassified as an independent species named *M sibonii*. In agreement with this new taxonomy, *M morganii* and *M sibonii* differed in terms of phylogenetic and biochemical characteristics, such as trehalose assimilation.

The second and main subpopulation included 215 isolates and corresponded to *M morganii* subspecies *morganii*.

A third subpopulation included 31 isolates that formed a phylogenetically independent, previously undefined group, with Mash distances of 94–95% with *M morganii* and *M sibonii* (figure 1, appendix pp 9–10, 23–32). We propose that this subpopulation be classified as a new subspecies of *M morganii* named *M morganii* subspecies *intermedius*.

Finally, one isolate (GenBank accession number NRQY0000000) was distinct from the four other populations (<94% ANI). This isolate was recovered from a grass grub (*Costelytra* spp) in New Zealand.

As shown by the phylogenetic tree, *M morganii* subspecies *morganii*, which includes most clinical isolates, is characterised by a wide genetic diversity (figure 1). However, five further important clusters (I–V) could be identified within *M morganii* subspecies *morganii* on the basis of their prevalence and their close genetic relationships.

Cluster I, which includes the NDM-1-producing isolates of the French outbreak (n=39), also includes unrelated carbapenemase-producing isolates from Germany (n=4), the UK (n=2), France (n=2), and Belgium (n=1), indicating that cluster I has already disseminated in Europe. A subtree and an SNP matrix constructed with isolates of cluster I showed that this cluster can be divided into three independent clusters (clusters IA, IB, and IC; figure 2). Within

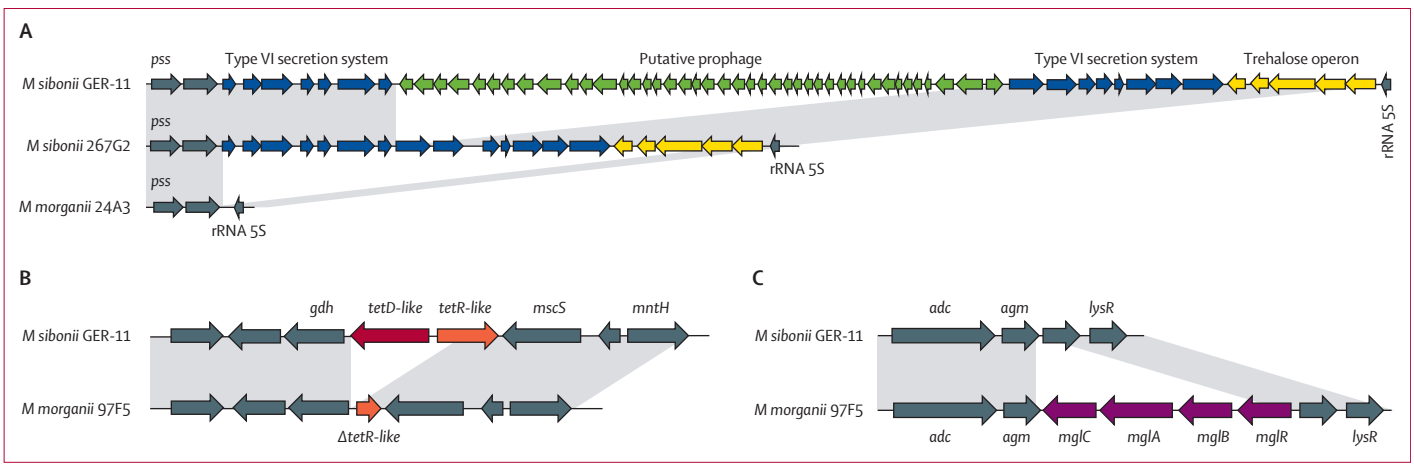


Figure 3: **Examples of specific genomic regions identified in *Morganella spp***

(A) Schematic representation and comparison of the trehalose operon locus in *M sibonii* GER-11, *M sibonii* 267G2, and *M morganii* subspecies *morganii* 24A3. Coding sequences are represented by arrows. A putative prophage is indicated in green, the trehalose operon in yellow, type VI secretion systems in blue, and other genes in black. (B) Schematic representation of the *tetD* locus in *M morganii* subspecies *morganii* GER-11 and *M sibonii* 97F5. The *tetD*-like gene is represented in red and its regulator (*tetR*-like) in orange. (C) Schematic representation of the putative galatactose or methylgalactoside ABC transporter *mgl* locus in *M morganii* subspecies *morganii* and *M sibonii*. In all panels, nucleotide sequences with ≥99% identity are highlighted in grey.

cluster IC, the SNP matrix showed that all isolates had fewer than 25 SNPs (between one and 23 SNPs), except for isolate 126H7, which had more than 35 SNPs. One French isolate, 81B3, did not belong to the main French cluster (cluster IC). This strain was part of cluster IB, which also included four German (GER-18, GER-19, GER-20, and GER-23) and two English (ANG-17 and ANG-18) isolates.

To investigate the evolution of cluster I, we calculated a molecular clock based on the nucleotide diversity of the core genome. The reference genome sequence belonged to the first isolate from the French outbreak (isolate 24A3). The number of genome-wide nucleotide substitutions was correlated with isolation dates to construct a time-scaled phylogeny. A root-to-tip regression analysis gave an R^2 of $4{\cdot}38\times10^{-4}$ (appendix p 11), which is probably due to the low number of isolates. The evolutionary rate was estimated at $7{\cdot}782\times10^{-7}$ substitutions per site per year (95% highest posterior density $3{\cdot}252\times10^{-7}$ to $2{\cdot}386\times10^{-6}$) corresponding to 3·12 SNPs per genome per year (95% highest posterior distribution 1·38–9·56). The last common ancestor between all strains belonging to cluster I was estimated to have been around 1972. If only isolates from the French outbreak are considered, the appearance was estimated to have been at the beginning of the 21st century (around 2001; appendix p 12).

Apart from cluster I, which was overrepresented due to the French outbreak, four additional prevalent clusters were evidenced in our collection (figure 1). We analysed the subtree of each cluster and compared the results with epidemiological data (date of isolation and suspicion of outbreak) obtained for each laboratory providing isolates (appendix pp 13–16). Cluster III is mostly composed of KPC-producers from the Czech Republic that have been suspected to be part of an outbreak. We identified several cross-country disseminations for each of the main clusters. For instance, within cluster IV we identified five isolates in the Czech Republic that carry either bla_{OXA-48} (TCH15 54 294) or bla_{KPC-2} (TCH3 42 744 and TCH9 48 659) and isolate TCH2 37590 that did not carry any carbapenemase; we also identified a close relationship between three isolates from France and Belgium (156C10, 249E6, and BEL-22).

We compared the core genomes of *M sibonii* (n=19, after quality control, including 2744 core genes) and *M morganii* subspecies *morganii* (n=209, including 2760 core genes) to identify unique features and to confirm results obtained with whole-genome phylogeny. 2697 genes were present in 95% of the strains in the whole genome dataset (comprising strains of both species), whereas 47 genes were specific to *M morganii* subspecies *morganii* and 63 were specific to *M sibonii* (appendix pp 33–36).

The biochemical characterisation of all isolates in our collection confirmed that all *M sibonii* isolates were able to use trehalose as a unique carbon source, whereas *M morganii* subspecies *morganii* strains were not able to. Among *M sibonii*-specific genes, we identified an operon putatively involved in the transport of trehalose (figure 3A). We cloned and expressed this entire operon from *M sibonii* into an *M morganii* subspecies *morganii* strain and conferred the ability of this strain to use trehalose as sole carbon source, demonstrating the functionality of this operon. The putative role of each component of this operon in trehalose use in *M sibonii* is summarised in appendix pp 17–18. Immediately downstream of the trehalose operon, we found a putative type VI secretion system in all *M sibonii* isolates (figure 3A).

M morganii subspecies *morganii* is intrinsically susceptible to tetracycline. By contrast, *M sibonii* has a *tetD*-like resistance gene and its *tetR* regulatory gene in its core genome (figure 3B). By expressing the *tetD*-like gene in *E coli*, we confirmed that this gene was able to confer resistance to tetracycline (MIC >128 mg/L) but not to tigecyline (MIC <0·25 mg/L) or eravacycline (MIC <0·25 mg/L).

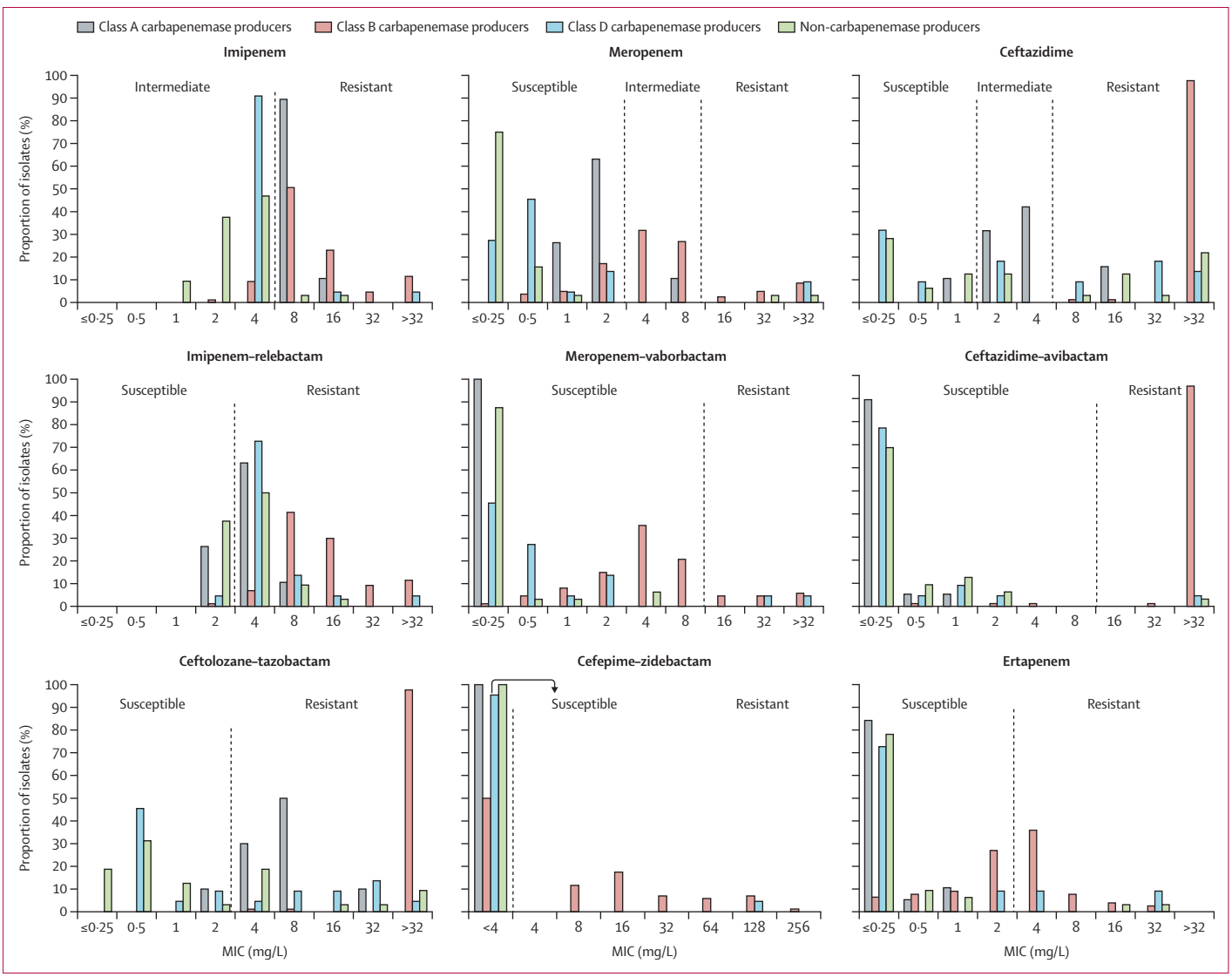


Figure 4: **MIC distribution of nine last-resort antimicrobials or antimicrobial combinations**
Minimum inhibitory concentration distributions were separated according to carbapenemase content and are indicated on the histograms. Clinical breakpoints correspond to European Committee on Antimicrobial Susceptibility Testing guidelines. MIC distribution for last-generation cyclines are shown in appendix p 19. MIC=minimum inhibitory concentration.

Another operon involved in methylgalatoside uptake was also identified (figure 3C). However, its role remains to be determined.

Susceptibility to several antibiotics, including three carbapenems, five new β-lactams–β-lactamase inhibitor combinations, and two last-resort cyclins was tested on isolates from the French and European NRC, the isolate from Canada, two isolates from Pasteur's Institute collection, and two susceptible isolates from Bicêtre Hospital (172 isolates in total). MIC distributions of nine antimicrobials or antimicrobial combinations are shown in figure 4 and appendix p 19; susceptibility percentages are summarised in the appendix (pp 37–39). Moderate susceptibility to imipenem was observed independently of carbapenemase production, because of the natural decreased susceptibility to imipenem in the *Morganella* genus. The combination of imipenem with relebactam did not substantially increase the efficiency of imipenem. Vaborbactam strongly restored meropenem susceptibility for all class A KPC-like enzyme-producing isolates, but this association showed a moderate effect on class D carbapenemases and no effect on class B MBL-producing isolates. The distribution of MICs of ceftazidime was heterogeneous except for MBL producers, which remained highly resistant. 19 (100%) of 19 class A carbapenemase producers, 21 (95%) of 22 class D carbapenemase producers, and 31 (97%) of 32 non-carbapenemase producers were susceptible to ceftazidime–avibactam. Conversely, all MBL producers were fully resistant to ceftazidime–avibactam. The MICs of temocillin showed a bimodal distribution with 69 (79%) of 87 MBL producers

and 22 (100%) of 22 class D carbapenemase producers being highly resistant (appendix pp 37–49). MICs of cefepime–zidebactam for most isolates were below the resistance threshold (≤4 mg/L). 19 (100%) of the 19 class A carbapenemase producers, 21 (95%) of 22 class D carbapenemase producers, and 32 (100%) of 32 non-carbapenemase producers were susceptible to cefepime–zidebactam, whereas only 43 (49%) of 87 MBL-producing isolates were susceptible to cefepime–zidebactam. The aztreonam–avibactam combination was tested in 50 MBL producers, of which 40 (80%) isolates had a MIC of 0·25 mg/L, nine (18%) of 0·5 mg/L, and one (2%) of 1 mg/L.

To decipher the structure of lipid A, a MALDIxin test was performed on ten colistin-resistant isolates (MIC >256 mg/L) and two isolates exhibiting susceptibility to polymyxin (BEL-5, with a MIC of colistin of 2 mg/L, and BEL-6, with a MIC of 0·5 mg/L). The test identified a strong decrease in L-ARA4N-modified lipid A in the susceptible isolates (BEL-5 and BEL-6; appendix p 20), suggesting that resistance to polymyxin is associated with addition of L-ARA4N on *Morganella* lipid A.[28] In isolate BEL-5, colistin susceptibility was possibly due to the insertion of *IS10R* immediately upstream the *arn* operon, leading to truncation of the PmrA–QseB binding site, which is likely to have modified the expression of the *arn* operon (appendix pp 21–22). In isolate BEL-6, the PmrA–QseB binding site was intact, but SNPs were detected in the *arn* operon that might affect the efficacy to bind L-ARA4N on lipid A (appendix p 22). However, the role of each mutation remains to be elucidated.

Discussion

In this study, among all *M morganii* isolates with reduced susceptibility to ertapenem or meropenem collected in France in 2013–21 by the NRC, we identified a subset of NDM-1-producing *M morganii* with very similar genetic backgrounds (<20 SNPs accumulated over 8 years), which we considered to be linked to a longitudinal outbreak. In *K penumoniae*, a cutoff at 20 SNPs per genome was established to decipher isolates from an outbreak,[29] and we think that this cutoff value applied to the whole genome of *M morganii* isolates alongside epidemiological data might be used to discriminate outbreak-related strains from non-clonally related *M morganii* isolates.

In our genomic analysis of *Morganella* spp isolates from France, other European countries, and Canada combined with whole-genome sequences available on GenBank, we used multiple approaches—two phylogenetic reconstructions and Mash distance analysis. On the basis of our results, we propose a modified taxonomy for the *Morganella* genus, including four species: *M psychrotolerans, M sibonii, M morganii,* and a new species represented by a unique strain. In addition, we propose that *Morganella morganii* be split into two subspecies named *M morganii* subspecies *morganii* and *M morganii* subspecies *intermedius*. Many traits, including an intrinsic resistance profile to tetracycline, the conservation of the ability to assimilate treahalose, or the presence of specific secretion systems (eg, a specific type VI secretion system), are associated with this novel taxonomic organisation.

In this study, the analysis of two *Morganella* isolates that were susceptible to colistin allowed us to show that intrinsic addition of L-ARA4N on lipid A via expression of *arnB-CADTEF* confers colistin resistance. Additionally, antimicrobial susceptibility testing identified antibiotics or antibiotic combinations to which isolates were most susceptible and which might, therefore, represent viable therapeutic options for the treatment of infections caused by multidrug-resistant *Morganella* spp. On the basis of their susceptibility profiles, we found that ceftazidime–avibactam, meropenem–vaborbactam, and cefepime–zidebactam could be considered as treatment options for class A and D carbapenemase-producing isolates and non-carbapenemase producers. Zidebactam is a β-lactamase inhibitor of the diazabicyclooctane family and is used in combination with cefepime to increase β-lactam efficiency.[30] In addition to its inhibition properties towards class A and class D β-lactamases, zidebactam has intrinsic antimicrobial activity through its binding to PBP-2 and PBP-3.[31] However, when used on its own, this molecule does not show any activity against the *Morganella* genus.[28] As observed with other Enterobacterales,[32] the novel β-lactamase inhibitors (avibactam, relebactam, vaborbactam, and zidebactam) do not restore the activity of carbapenems (imipenem or meropenem) or broad-spectrum cephalosporins (ceftazidime, cefepime) in MBL-producing *Morganella* spp isolates.

This study has some limitations. Despite the analysis of 270 *M morganii* and *M sibonii* genomes, only a small number of isolates were involved in outbreaks (the French outbreak and putative Czech outbreak), making it difficult to definitively assess whether the 20 SNPs cutoff we used to differentiate outbreak isolates is robust. Additionally, we used Illumina technology for genome sequencing, which does not allow reconstruction of large genetic structures such as plasmids.

In conclusion, we analysed a large collection of *Morganella* spp isolates, leading to a proposed modified taxonomy of *Morganella* based on identification of key specific phenotypes (eg, trehalose assimilation and tetracycline resistance). Regarding antimicrobial resistance, *Morganella* spp have the potential to become a threatening issue in future.[33] Accordingly, more comprehensive studies should be carried out on *Morganella* spp and their ability to acquire resistance. Such studies should aim to improve knowledge of the dissemination of high-risk clones such as the one responsible for the 2013–21 outbreak in France (appendix p 2) and that has already spread to other European countries.

Contributors

RAB: conceptualisation, data curation, investigation, supervision of the research, data verification, writing original draft, and writing, reviewing, and editing. EC, AP, DG, CE, ABJ, MD, AJ, and GL-M: investigation and analysis. KH, PB, YG, NP, MG, APAH, KvdZ, PA, RH, VS, and JH: biological resources, review, and editing. EPCR: investigation, analysis, and

writing, reviewing, and editing. TN: data verification, funding, reviewing, and editing. LD: conceptualisation, data curation, investigation, supervision of the research, data verification, writing original draft, and writing, reviewing, and editing. All authors had full access to all the data in the study and accept responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

Genomes of *Morganella* spp isolates were deposited in the GenBank database in bioproject PRJNA996528.

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