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Supplementary material

Supplementary Material is available in the ScienceData folder: https://sciencedata.dk/shared/2963696a552f6084b776f60fadc4dc3c.

The folder contains:

- One Microsoft Excel file (D5.5. Supplementary Material) containing the sequencing details and quality control results for the sequence data, as well as all the outputs from the bioinformatics analyses performed for this report;
- The three databases of genes and point mutations that were analysed in this report (3 Microsoft Excel files: AMRFinderPlus, CARD-RGI and ResFinder);
- All genomic data used in this report (70 sequence files).

List of abbreviations

Aba: *Acinetobacter baumannii*
AFP: AMRFinderPlus
AMR: Antimicrobial resistance
ARG: Antimicrobial resistance gene
AST: Antimicrobial susceptibility testing
BLDB: Beta-lactamase Database
BLI: β-lactamase inhibitor
CCRE: Carbapenem- and/or colistin-resistant Enterobacterales
CLI: Command-line interface
CR: CARD-RGI
CRAb: Carbapenem- and/or colistin-resistant *Acinetobacter baumannii*
CRE: Carbapenem-resistant Enterobacterales
CRPa: Carbapenem- and/or colistin-resistant *Pseudomonas aeruginosa*
DB: Database
EARS-Net: European Antimicrobial Resistance Surveillance Network
ECDC: European Centre for Disease Prevention and Control
Eco: *Escherichia coli*
ENA: European Nucleotide Archive
EQA: External quality assessment
EU/EEA: European Union/European Economic Area
EUCAST: European Committee on Antimicrobial Susceptibility Testing
EURGen-Net: European Antimicrobial Resistance Genes Surveillance Network
EURGen-RefLabCap: European Antimicrobial Resistance Genes Surveillance - Reference Laboratory Capacity
EURL: European Union Reference Laboratory
EURL-PH-AMR: European Union Reference Laboratory for Public Health on Antimicrobial Resistance in Bacteria
HaDEA: European Health and Digital Executive Agency
HMM: Hidden Markov model
I: Suceptible, increased exposure
Kpn: *Klebsiella pneumoniae*
LR: Long-read
NCBI: National Center for Biotechnology Information
NRL: National Reference Laboratory
Pae: *Pseudomonas aeruginosa*
PBP: Penicillin binding protein
PCR: Polymerase chain reaction
PH: Public health
PM: Point mutation
PT: Proficiency test
qPCR: Real-time polymerase chain reaction
R: Resistant
RF: ResFinder
RRA: Rapid Risk Assessment
SR: Short-read
WGS: Whole-genome sequencing

Antimicrobial abbreviations
AMK: Amikacin
AZA: Aztreonam-avibactam
CIP: Ciprofloxacin

COL: Colistin
CZA: Ceftazidime-avibactam
CZT: Ceftolozane-tazobactam
FDC: Cefiderocol
GEN: Gentamicin
IMR: Imipenem-relebactam
IPM: Imipenem
LVX: Levofloxacin
MEM: Meropenem
MEV: Meropenem-vaborbactam
MFX: Moxifloxacin
TOB: Tobramycin

1. Introduction

The primary role of the European Union Reference Laboratory (EURL) for Public Health (PH) on Antimicrobial Resistance (AMR) in Bacteria (EURL-PH-AMR) is to provide support to the European Centre for Disease Prevention and Control (ECDC) and its associated disease networks: the European Antimicrobial Resistance Surveillance Network (EARS-Net)[1] and the European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net)[2]. The key focus is to increase the capability and capacity of the members of these networks for provision of reliable and comparable microbiology laboratory data, for national and European-level surveillance in the field of AMR.

The Grant Agreement no. 101194806 for the EURL-PH-AMR specifies that the EURL will perform the testing and review of bioinformatic tools and reference functional gene databases and provide recommendations on surveillance of carbapenem- and/or colistin-resistant Enterobacterales (CCRE), *Pseudomonas aeruginosa* (CRPa) and *Acinetobacter baumannii* (CRAb) for EURGen-Net.

This document reports on the identification, testing and results of different bioinformatics tools and databases for surveillance of AMR in the target pathogens. It includes the rationale for selection of specific antimicrobials and respective genetic determinants of AMR, as well for the selection of three bioinformatics tools and databases to be tested (AMRFinderPlus, CARD-RGI and ResFinder). It makes use of a genomic dataset of 18 bacterial strains represented by multiple types of sequence data and file formats to test the tools, and it describes the differences identified across the databases and the outputs from the analyses. Finally, the document provides specific recommendations on which genetic determinants of resistance to investigate within the context of EURGen-Net, as well as the optimal bioinformatics strategies to be applied in order to monitor those genes or chromosomal mutations for surveillance purposes.

This is the first version of this report, created in 2026. The EURL-PH-AMR plans to create a second version in 2028, and a third version in 2030. Considerations for these updates are presented in this report.

[1] https://www.ecdc.europa.eu/en/about-us/networks/disease-networks-and-laboratory-networks/ears-net-data
[2] https://www.ecdc.europa.eu/en/about-us/who-we-work/disease-and-laboratory-networks/EURGen-net

2. Technical and scientific rationale

2.1. European surveillance of antimicrobial resistance and identification of important genetic determinants of resistance

Between 2018 and 2025, ECDC has investigated cross-border outbreaks of multidrug-resistant pathogens and emerging AMR, resulting in the publication of Rapid Risk Assessment (RRA) or other scientific outputs to guide public health action [1,2]. The results of those EURGen-Net investigations across the years included cross-border spread and clusters of *Klebsiella pneumoniae* carrying *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$, multi-country clusters of *Escherichia coli* carrying *bla*$_{\text{OXA-244}}$, and emergence of high-risk lineages of *E. coli* carrying *bla*$_{\text{NDM-5}}$.

Dedicated efforts are in place within EURGen-Net to characterize the genomic epidemiology of the target pathogens, through ECDC's European genomic surveys. Specifically, these are the CCRE 2019 survey (finalised), the 2025 carbapenem-resistant Enterobacterales (CRE) survey (ongoing), and the CRAb 2024 survey (ongoing) [3–5]. Results from the ECDC's 2019 CCRE genomic survey highlighted an increase of *E. coli* carrying *bla*$_{\text{NDM-5}}$, and also suggested that *K. pneumoniae* carrying *bla*$_{\text{KPC-2}}$ and *bla*$_{\text{VIM-1}}$ were spreading in Europe [1,6]. In carbapenem-resistant (R) or carbapenem-susceptible, increased exposure (I) *K. pneumoniae* the most prevalent carbapenemases were *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC-3}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{KPC-2}}$ and *bla*$_{\text{VIM-1}}$; in *E. coli* these were *bla*$_{\text{NMD-5}}$, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$. The survey also found that chromosomal point mutations (PM) affecting colistin susceptibility were more common than *mcr* genes in carbapenem-R/I *K. pneumoniae*, although the inverse situation was seen for *E. coli*. Approximately 14% of *K. pneumoniae* and 9% of *E. coli* carbapenem-R/I isolates harboured 16S rRNA methyltransferases (specifically *armA* and *rmt* genes). More recently, preliminary results from the CRE25 survey show a decrease of *bla*$_{\text{KPC}}$ but increase in *bla*$_{\text{NDM}}$ (especially *bla*$_{\text{NMD-1}}$ and *bla*$_{\text{NDM-5}}$) for carbapenem-R/I *K. pneumoniae*. In *E. coli*, *bla*$_{\text{OXA-48-like}}$ carbapenemases increased (especially *bla*$_{\text{OXA-244}}$), and there were slight decreases in the proportions of *bla*$_{\text{NDM}}$ and *bla*$_{\text{KPC}}$ [7].

In June 2025 the EURL-PH-AMR conducted the e-survey 'Survey of capacity for National Reference Laboratory (NRL) core functions and collaborative research activities in EURGen-Net'. It contained questions related to the methods routinely used by the NRLs for phenotypic and genotypic characterisation of AMR, status of implementation of whole-genome sequencing (WGS), and other related topics. The EURL received replies from 33 NRLs, and 31 of those reported to perform some type of genotypic characterisation of AMR. The methods used in their settings were WGS (n=26), polymerase chain reaction (PCR) (n=19), real-time PCR (qPCR) (n=12), and single gene sequencing (n=5). Five NRLs further reported 'other' methods, including loop-mediated isothermal amplification (n=3), recombinant enzyme polymerase amplification (n=1), NG-TEST® CARBA-5® (NG Biotech SAS, France) (n=1), and BioFire® FilmArray® (bioMérieux SA, France) (n=1).

Out of the 31 NRLs that perform genotypic characterisation of AMR, all of them routinely test for the presence of *bla*$_{\text{VIM}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-48-like}}$ β-lactamase genes, but only 28 laboratories test for *bla*$_{\text{IMP}}$. Twenty-four laboratories test for *mcr* genes, but only 11 test for PMs associated with colistin resistance. In addition to the genetic determinants that could be directly selected as multiple-choice options in the survey, 21 participants reported to test for other AMR mechanisms:

- *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-51}$, *bla*$_{OXA-58}$ and *bla*$_{OXA-72}$ for *A. baumannii*
- *ompK* porin alterations for *K. pneumoniae*
- Other β-lactam resistance mechanisms, specifically *bla*$_{CTX-M}$, *bla*$_{GES}$, *bla*$_{SME}$, *bla*$_{IMI}$, *bla*$_{GIM}$, *bla*$_{SIM}$, AmpC β-lactamases, and PMs (e.g. in penicillin-binding proteins or other PMs associated with overexpression of β-lactamases)
- PMs associated with cefiderocol resistance
- Genes and PMs associated with fluoroquinolone resistance
- 16S rRNA methylases
- Other mechanisms outside of the scope of this report.

The results show that capacity and individual approaches for WGS-based surveillance are not homogeneous within Europe. It is important that genomic results can be directly compared between settings, allowing all data to be combined to create actionable information. Moreover, any catalogue of genetic determinants to be used for surveillance purposes should align with current findings from European genomic surveillance studies.

Ideally, it should also be possible to associate genomic data with results from phenotypic surveillance. To make this possible, the AMR genes (ARG) and chromosomal PMs being monitored should be representative of the most relevant resistance phenotypes under European surveillance. Phenotypic surveillance of AMR at European level includes information collected by EARS-Net. Every year, countries submit antimicrobial susceptibility data on eight invasive pathogens, including *E. coli*, *K. pneumoniae*, *Acinetobacter* spp. and *P. aeruginosa* [8]. Surveillance covers multiple antimicrobial classes including colistin, fluoroquinolones, aminoglycosides and β-lactams. Within the β-lactam class there is a focus on carbapenems, as well as third- and fourth-generation cephalosporins. Recently, novel antimicrobial agents have been included, specifically carbapenems and cephalosporins in combination with β-lactamase inhibitors (BLI), aztreonam-avibactam, and cefiderocol.

One question in the EURL-PH-AMR e-survey focused on the phenotypic antimicrobial susceptibility testing (AST) performed at the NRLs, and the replies are summarised in **Table 1**. Almost all (n=32) NRLs reported to use at least one AST method in their setting, with one participant replying that they only perform genomic analyses. Most NRLs perform AST of carbapenems and colistin for all pathogens (≥85%), whereas AST of the novel agents is not implemented as broadly.

Table 1. *Number and percentage of the 32 National Reference Laboratories in Europe that reported performing reference phenotypic antimicrobial susceptibility testing, per antimicrobial and per pathogen*

Pathogen	**Enterobacterales**		***A. baumannii***		***P. aeruginosa***	
Antimicrobials	**N**	**%**	**N**	**%**	**N**	**%**
Carbapenems	31	96.9	30	93.8	30	93.8
Carbapenems with BLI	22	68.8	16	50.0	18	56.3
Colistin	30	93.8	28	87.5	28	87.5
Ceftazidime-avibactam	27	84.4	17	53.1	26	81.3
Ceftolozane-tazobactam	19	59.4	14	43.8	20	62.5
Cefiderocol	21	65.6	21	65.6	21	65.6
Aztreonam-avibactam	14	43.8	8	25.0	9	28.1
Other [a]	8	25.0	8	25.0	7	21.9

BLI: β-lactamase inhibitor.

[a] *Tigecycline (n=5), minocycline (n=2), eravacycline (n=1), fosfomycin (n=3), sulbactam-durlobactam (n=2), ampicillin-sulbactam (n=1), cefepime-enmetazobactam (n=1), cephalosporins (n=1), plazomicin (n=1), aminoglycosides (n=2), fluoroquinolones (n=1).*

The genetic determinants of resistance reported by participants in the e-survey captured some of the relevant mechanisms of AMR towards carbapenems, newer β-lactam combinations, cefiderocol, and colistin. Other genes or PMs could be monitored to ensure greater coverage of surveillance efforts, specifically those associated with resistance towards aminoglycosides and fluoroquinolones. This would also ensure alignment with phenotypic surveillance programmes and with the NRLs' current practices for phenotypic AST and genotypic analyses.

Table 4 in Section 5 of this report presents the EURL-PH-AMR proposal for the minimum catalogue of ARGs and chromosomal PMs to be monitored for the purpose of AMR surveillance in Europe.

Several of the β-lactamases included in **Table 4** may present different phenotypes depending on the specific variant (*bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{OXA}$). A useful resource is the Beta-lactamase Database (BLDB)[3], which provides the expected phenotypes for each variant [9].

Importantly, the detection of ARGs or chromosomal PMs should not be used to infer phenotypic AST profiles. WGS is an invaluable resource for monitoring the emergence, distribution and evolution of bacterial clones and populations, for detecting resistance and virulence mechanisms and mobile genetic elements, and for aiding in the investigation of outbreaks. However, the presence or absence of specific genetic determinants cannot, by itself, be used as an alternative for phenotypic AST results. Detailed considerations on the topic are available in the 2025 European Committee on Antimicrobial Susceptibility Testing (EUCAST) update on the role of WGS in antimicrobial susceptibility prediction of bacteria [10].

2.2. Identification of target bioinformatics tools and databases

There is a large variety of bioinformatics tools available for detection of ARGs and chromosomal PMs. These tools vary in the file formats and type of data that can be submitted: raw data as fastq files and/or assemblies as fasta files, originating from short-read (SR) or long-read (LR) sequencing. Moreover, the tools can be commercial or open-source, web-based or command-line interface (CLI) tools, and importantly they can be frequently curated or not.

Members of the EURL-PH-AMR consortium were the contractors for the European Health and Digital Executive Agency (HaDEA) four-year project 'European Antimicrobial Resistance Genes Surveillance - Reference Laboratory Capacity' (EURGen-RefLabCap)[4] [11]. The project provided networking and technical support activities to the PH NRLs/national expert laboratories in 37 European countries. The priority pathogens in the project were those within the scope of EURGen-Net, specifically healthcare-associated CCRE, CRPa and CRAb. The project included three genomic proficiency tests (PTs) in 2022 [12], 2023 [13] and 2024 [14].

Annex 1 presents the information on bioinformatics tools and databases for

[3] http://www.bldb.eu/

[4] https://www.eurgen-reflabcap.eu/

detection of AMR reported by the 34 European laboratories that participated in the 2022-2024 EURGen-RefLabCap genomic PTs. The three tools/databases most commonly used were:

- ResFinder[5] (n=28)
- AMRFinderPlus[6] (n=21) (including 4 laboratories applying its database with the commercial software Ridom SeqSphere+[7], and 3 laboratories that used a mix of AMRFinderPlus and Ridom SeqSphere+ software)
- CARD-RGI[8] (n=8).

Almost all the other tools reported by the participants make use of the databases associated with ResFinder, AMRFinderPlus and/or CARD-RGI (for example ABRicate, abriTAMR, ARIBA, Kleborate, staramr).

Most laboratories used open-source tools (even if in combination with commercial software), with only one participant exclusively using commercial tools. Between 8 and 10 participants exclusively used web-based bioinformatics tools.

EURGen-RefLabCap published protocols for European WGS and bioinformatics for PH surveillance of CCRE, CRAb and CRPa, which recommended the use of open-source tools with frequently curated databases [15,16]. The three tools most frequently reported by the laboratories in the genomic PTs align with those recommendations. There are other bioinformatics tools that fit those criteria, with the main limitation of being more specific for certain pathogens. These may be considered for future updates of this report (section 6).

Moreover, the analysis plan for ECDC's CRE25 survey genomic data describes the standard WGS pipeline implemented at ECDC. This includes the use of ResFinder database for *E. coli* and the use of Kleborate for *K. pneumoniae* (which relies on CARD database v3.2.9 for the species) [1,17]. ECDC's plan to analyse CRAb genomes follows EURGen-RefLabCap recommendations [5].

Within this context, the EURL-PH-AMR selected AMRFinderPlus, CARD-RGI and ResFinder as the bioinformatics tools and databases to be tested for this report. Their attributes are summarised in **Table 2**.

[5] https://genepi.food.dtu.dk/resfinder
[6] https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/
[7] https://www.ridom.de/seqsphere/ug/v90/Overview.html
[8] https://card.mcmaster.ca/analyze/rgi

Table 2. *Attributes of the bioinformatics tools and databases tested for this report including the specific versions used for the tests*

Tools	Accepted file formats [a]	Tool versions	DB versions	Links
AMRFinderPlus	fasta	4.2.7	2026-03-24.1	CLI: https://github.com/ncbi/amr DB: https://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/AMRFinderPlus/database/latest/
CARD-RGI	fasta	RGI 6.0.5	CARD 4.0.1	CLI: https://github.com/arpcard/rgi Webtool: https://card.mcmaster.ca/analyze/rgi DB: https://card.mcmaster.ca/download#download-software
ResFinder	fasta and fastq	4.7.2	ResFinder 2.5.1 PointFinder 4.1.1	CLI: https://github.com/genomicepidemiology/resfinder Webtool: https://genepi.food.dtu.dk/resfinder ResFinder DB: https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/ PointFinder DB: https://bitbucket.org/genomicepidemiology/pointfinder_db/src/master/

CLI: command-line interface; DB: database.

[a] *All tools accept both short- and long-read sequencing files.*

3. Testing strategy

3.1. Genomic dataset

The three EURGen-RefLabCap genomic PTs provided to European NRLs included sequence data or biological material from 12 strains. These provided a strong basis for the genomic dataset used for testing the bioinformatics tools analysed in this current report, since they were specifically selected within the EURGen-Net framework and therefore included genetic determinants of AMR relevant to European epidemiology. The raw sequence data generated for those genomic PTs are published on the European Nucleotide Archive[9] (ENA). However, that dataset lacked some of the genetic determinants of AMR proposed by the EURL-PH-AMR for European genomic surveillance (**Table 4**). Therefore, the collection was complemented by recovering additional genomes from ENA or from the National Center for Biotechnology Information[10] (NCBI). This ensured full coverage of the gene families and chromosomal PMs included in this report.

The complete dataset used for testing the bioinformatics tools included 18 strains, incorporating all three types of pathogens, specifically CCRE (n=11, including 4 *E. coli* and 7 *K. pneumoniae*), CRAb (n=3) and CRPa (n=4). Data types (SR and LR) and input file formats (fastq and fasta) were varied within the genomic dataset. For the 12 strains originating from EURGen-RefLabCap PTs, both raw data and assemblies from both SR and LR sequencing were included in the analysis. For the remaining 6 strains, publicly available SR sequencing data were used (fasta files for 4 strains, and fastq files for 2 strains which were complemented by assemblies produced at DTU Food).

Annex 2 presents the characteristics of the 18 test strains and sequences.

All sequence data, the respective sequencing details, and quality control results are provided as Supplementary Material in ScienceData: https://sciencedata.dk/shared/2963696a552f6084b776f60fadc4dc3c.

3.2. Testing strategy

The databases that support the three bioinformatics tools were downloaded, and screened to identify if all the relevant genetic determinants of AMR included in this report were present (n=64). Moreover, it was investigated if the expected phenotypes were associated with the ARGs and PMs.

Acquired ARGs or gene families (n=54) were defined as either present or absent from the databases, and the assigned *in silico* phenotypes were evaluated. The following genes were analysed: *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, specific variants of *bla*$_{GES}$ (*bla*$_{GES-4}$, *bla*$_{GES-5}$, *bla*$_{GES-14}$), *bla*$_{OXA-48-like}$ (*bla*$_{OXA-48}$, *bla*$_{OXA-162}$, *bla*$_{OXA-181}$, *bla*$_{OXA-204}$, *bla*$_{OXA-232}$, *bla*$_{OXA-244}$), *bla*$_{OXA-655}$, *bla*$_{OXA-198}$, *bla*$_{OXA-24/40-like}$ (*bla*$_{OXA-24/40}$, *bla*$_{OXA-25}$, *bla*$_{OXA-26}$), *bla*$_{OXA-23-like}$ (*bla*$_{OXA-23}$, *bla*$_{OXA-27}$, *bla*$_{OXA-49}$), *bla*$_{OXA-58}$, *bla*$_{CMY}$, *mcr* (*mcr-1* to *mcr-10*), *qnr* (*qnrA*, *qnrB*, *qnrS*, *qnrVC*), *qepA*, *armA*, *rmt* (*rmtA* to *rmtH*), *ant(2'')-Ia*, *aph(3')-VI*, *aac(3)-I*, *aac(3)-II*, *aac(6')-I*, *aac(6')-Ib-cr*, *aac(6')-II*.

[9] https://www.ebi.ac.uk/ena/browser/home
[10] https://www.ncbi.nlm.nih.gov/

Chromosomal genes that may contain PMs associated with AMR (n=10) were evaluated as present or absent, if the gene existed specifically as a target for PMs, which PMs were listed, and which *in silico* phenotypes were predicted. The genes were: *ftsI, pmrA, pmrB, phoP, phoQ, mgrB, gyrA, gyrB, parC, parE.*

All sequence data were submitted to the three bioinformatics tools using CLI, as well as webtool if available (CARD-RGI and ResFinder). Species were defined when allowed (AMRFinderPlus and ResFinder). The three tools use different sequence search strategies. AMRFinderPlus combines BLASTP-based protein alignment with hidden Markov model (HMM) profile search, and uses translated alignment when nucleotide input is provided, which improves the detection of divergent and truncated alleles and allows it to flag premature stop codons. ResFinder uses k-mer alignment when raw reads are submitted, and BLAST when assemblies are submitted; in both cases the search is at the nucleotide level. ResFinder does not perform HMM-based protein search, which can limit the detection of divergent alleles at the family level. ResFinder also reports the closest named hit even when the underlying sequence is divergent from any named allele, which should be considered when interpreting novel variants. CARD-RGI uses BLAST or DIAMOND alignments against the CARD reference, with per-model curated bitscore cutoffs. These methodological differences may affect detection of low-coverage or divergent variants and were not formally evaluated in this report.

AMRFinderPlus CLI was run with the required argument --nucleotide, and the optional argument --organism. CARD-RGI and ResFinder webtool options were kept at their default values, with the same options for CLI:

- In CARD-RGI webtool the following options were selected: 'Perfect and strict hits only', 'Exclude nudge' and 'High quality/coverage'. In the CLI the following optional arguments were not added: --include_loose, --include_nudge, --low_quality.
- In ResFinder webtool 'Threshold for %ID' was 80% and 'Minimum length' was 60%. The respective CLI arguments were --threshold 0.8 and --min_cov 0.6. For both submission methods, the PointFinder database was selected according to the available bacterial species, specifically 'Escherichia coli' and 'Klebsiella' (CLI arguments --point and --species).

Bioinformatics results were analysed to determine if there were differences in the genetic determinants of resistance and predicted *in silico* phenotypes obtained with the three bioinformatics tools. These comparisons were essentially based on the results obtained with SR fasta files through CLI submissions, as that data type, file format and submission method were transversal to all test genomes and tools.

Furthermore, it was also determined if there were significant differences between types of sequence data and file formats (SR and LR, fastq and fasta files), and submission methods (CLI and web-based). This was achieved by comparing the intra-strain results obtained for each combination, when available.

The databases supporting the bioinformatics tools are available as Supplementary Material in ScienceData:
https://sciencedata.dk/shared/2963696a552f6084b776f60fadc4dc3c.

4. Results and discussion

4.1. Differences between the databases

The results described in this section refer directly to the database versions listed in **Table 2**. Since all three tools are frequently curated, it is expected that the specific genes and PMs included in each database and/or the respective *in silico* predicted phenotypes will change over time.

AMRFinderPlus contained all relevant acquired ARGs (n=54), and these were correctly associated with the antimicrobial classes affected by them. However, there were some limitations regarding the specific antimicrobials from each class listed in the database. It was **the most comprehensive database in regard to PMs**, however there were still some issues (**Table 3** and **Annex 3**).

In AMRFinderPlus output the field 'Class' states the antimicrobial class(es) affected by the genes or PMs, and the field 'Subclass' intends to list all antimicrobials from those classes that would be affected by the presence of the ARG or PM. However, the field 'Subclass' was very incomplete across the database. Often, that field only contained the same information as the field 'Class'. Most of the antimicrobials included in this report were not listed individually or were only listed very rarely. Moreover, individual antimicrobials were listed inconsistently across the different ARGs and PMs present in the database. Examples of these shortcomings were:

- Meropenem and imipenem only appeared five times in the full database, ceftazidime-avibactam appeared seven times, cefiderocol appeared 26 times, aztreonam-avibactam appeared 21 times. Carbapenems in combination with BLI and ceftolozane-tazobactam did not exist in the database. Therefore, these antimicrobials are considered absent from the database because they appear too infrequently and are not consistently linked to the relevant genetic determinants.
- Not all *bla*$_{KPC}$ presented 'Carbapenem' within the 'Subclass'; it was not evaluated if the variants without 'Carbapenem' correspond to those with reduced carbapenemase activity as described in BLDB.
- The ampC enzymes only listed 'Cephalosporin' as 'Subclass', without details.
- Different *aac(6')-I* entries listed different antimicrobials in the 'Subclass' (some contained only 'Aminoglycoside', others contained amikacin and tobramycin, and others listed other agents). *rmt* genes only listed 'Aminoglycoside' as 'Subclass', and *armA* and *aac(3)-II* listed gentamicin but lacked other expected aminoglycosides.
- Specific quinolones did not exist in the database. ARGs and PMs associated with quinolone resistance only listed 'Quinolone' in the 'Subclass' field.

The downloadable CARD database contained all relevant acquired ARGs (n=54). There were some issues in the association between genetic determinants and the expected *in silico* predicted phenotypes. **There were significant limitations regarding the chromosomal PMs** (**Table 3** and **Annex 3**).

The database only lists the antimicrobial class(es) for each ARG and PM (field 'Drug class'). The association with specific antimicrobials is performed by the RGI tool through the relationships between the ontology terms. The limitations in the 'Drug

class' field were the following:

- BLI did not exist in the database (with very minor exceptions), therefore are considered absent from the database.
- OXA-655 was missing carbapenems from the field, although the other OXA listed in this report included 'carbapenem'.
- All GES enzymes contained carbapenems within the field, although that would only be expected for certain variants.
- Colistin was listed as 'peptide antibiotic', although other fields included further information such as 'phosphoethanolamine transferase' or 'colistin resistance'. The terms Polymyxin(s) were not found in relation to any of the resistance determinants present in this report.

ResFinder databases contained almost all acquired ARGs (n=53). One ARG was absent, and there were other issues related with the expected phenotypes. **There were major limitations regarding chromosomal PMs**. Notably, the PointFinder database does not include options for *A. baumannii* nor *P. aeruginosa*, thus the tool cannot report species-specific chromosomal PMs for those organisms. There were other extensive problems identified within the PMs for *E. coli* and *K. pneumoniae* (**Table 3** and **Annex 3**).

ResFinder provides the fields 'Class' and 'Phenotype', showing the antimicrobial class(es) and the individual agents that are expected to be affected by the gene or PM. There were some limitations in the phenotypes listed in the database:

- BLI and cefiderocol did not exist in the database (with very minor exceptions), therefore are considered absent from the database.
- *bla*$_{\text{OXA-655}}$ was not present in the database.
- *bla*$_{\text{OXA-232}}$ listed several antimicrobials but not carbapenems.
- *bla*$_{\text{OXA-25}}$*, bla*$_{\text{OXA-26}}$*, bla*$_{\text{OXA-27}}$*, bla*$_{\text{OXA-49}}$ *and bla*$_{\text{OXA-244}}$ did not have assigned antimicrobials, instead showed 'Unknown Beta-lactam'.
- No *bla*$_{\text{GES}}$ variant contained carbapenems within the phenotype field.
- *qnr* and *qep* genes only contained ciprofloxacin in the phenotype field, without other fluoroquinolones.
- There were two *aph(3')-VI* variants with gentamicin listed in the phenotype.

Table 3. *Main limitations related to chromosomal PMs identified in each database*

Genes	**AMRFinderPlus**	**CARD-RGI**	**ResFinder** (Eco and Kpn only [a])
ftsI	-	No PMs for Eco, Aba, Pae. Minimal PMs for Kpn.	No PMs.
pmrA	No PMs for Eco.	No PMs.	No PMs for Kpn.
pmrB	-	No PMs.	No PMs for Kpn. Minimal PMs for Eco.
phoP (Kpn and Pae)	-	Fewer PMs than AFP.	No PMs.
phoQ (Kpn and Pae)	-	Fewer PMs than AFP.	No PMs.
mgrB (Kpn)	Lacks insertion sequences.	No PMs.	No PMs.
gyrA	-	No PMs for Kpn. Fewer PMs than AFP.	Slightly fewer PMs than AFP.
gyrB (Eco and Pae)	-	No PMs.	-

Genes	AMRFinderPlus	CARD-RGI	ResFinder (Eco and Kpn only [a])
parC	-	No PMs for Kpn. Fewer PMs than AFP.	Slightly fewer PMs than AFP.
parE (Eco and Pae)	-	Fewer PMs than AFP.	Slightly fewer PMs than AFP.

Aba: A. baumannii; AFP: AMRFinderPlus; Eco: E. coli; Kpn: K. pneumoniae; Pae: P. aeruginosa; PM: point mutations.
[a] ResFinder does not contain PMs databases for A. baumannii nor P. aeruginosa.

Conclusions

All of the databases had the limitation of not including BLI nor cefiderocol. A major weakness was the absence of chromosomal PMs for *A. baumannii* and *P. aeruginosa* in the PointFinder database, which substantially restricts its utility for the surveillance of those two pathogens. Additionally, more localized limitations were identified across the databases, including the absence of specific PMs, and the non-harmonised attribution of *in silico* predicted phenotypes to the ARGs and PMs described in this report. These are inconsistencies that carry direct implications for the comparability of results obtained with the different tools. Moreover, insufficient or incorrect predicted *in silico* phenotypes may affect the reporting of genes or PMs for surveillance purposes, or lead to under- or over-reporting of AMR determinants associated with specific antimicrobial classes.

Users of these databases and/or the respective bioinformatics tools should be familiar with the genetic determinants listed in each database, and understand that a negative result (absence of a gene or PM) may be a true false negative (i.e. reflect the absence of a gene or mutation from the database, while it is in fact present in the genome). Users should carefully evaluate the phenotypes predicted by each tool and confirm if the output is accurate and complete, and complement it based on expert knowledge or validated reference sources, such as this current report.

4.2. Differences between the outputs of the three bioinformatics tools

The overall identification of ARGs and PMs was limited by the specific genes and mutations included in each database, as described in section 4.1. For the genomic test collection included in this report, the main consequences of the **limitations in the databases were that no tools were able to detect certain PMs in *mgrB* (n=1), *parC* (n=1), *pmrB* (n=3), *phoP* (n=2) and *ftsI* (n=1)**.

In general, all three bioinformatics tools were able to identify all acquired ARGs to the same extent, with three exceptions:

- **ResFinder was unable to report one ARG.** It missed *aac(3)-IIg* in one strain [EURGen-2022-04]. This case was a true false negative, as users would expect that the tool would report the gene since *aac(3)-II* variants are part of its database. This limitation was observed for all data types, file input formats and submission methods. ResFinder was able to identify other variants of the gene in all the other strains that contained it.
- **CARD-RGI missed multiple genes in seven strains,** especially genes associated with aminoglycoside resistance. Most of those instances were true

false negatives, and a few were due to the genes being truncated at the end of the contigs:

- It missed *armA*, *ant(2")-Ia, aac(6')-Ib-cr* and *qnrS* in strain KR6077 (*qnrS* was truncated).
- It missed *aac(6')-Ib-cr* in strain 53_S27, *aac(6')-Ib* in strain Kpn-3-M28196 and *aac(6')-Ib* in strain 6717008. In the last two strains the gene was truncated, but not in the first case.
- It missed $bla_{\text{OXA-23}}$ and *ant(2")-Ia* in strain ABIsac_ColiR. Further considerations regarding this $bla_{\text{OXA-23}}$ are presented below.
- It missed *aac(6')-Ib-cr* in EURGen-2023-02 and *aac(6')-Ib10* in EURGen-2024-04. In both cases the genes were absent from the analyses performed with SR data, but present in results for LR data (section 4.3). Since the comparison of the three tools was based on SR fasta files submitted via CLI, these were considered as limitations. *aac(6')-Ib10* was identified by other tools as *aac(6')-Ib-cr* but identified as *aac(6')-Ib10* in other CARD-RGI outputs for the same strain (this was a general inconsistency observed across strains, see further details below).

- **CARD-RGI could not accurately report chromosomal PMs associated with fluoroquinolone resistance in *K. pneumoniae.*** It reported the correct amino acid changes in *gyrA* and *parC*, however these were reported as if being associated with *E. coli* or *Salmonella* wild-type sequences. This was observed for all strains that harboured those PMs (n=6), for all data types, file input formats and submission methods.

In other situations, identification of ARGs revealed **some inconsistencies in the gene variants and percentage of identity** reported by the three tools. These differences can likely be attributed to heterogeneous nomenclature and/or different reference sequences used in each of the databases, coupled with differences in the searching strategy used by each tool. In practical terms, most of these discrepancies had no impact in the ARG families reported by the tools. Importantly, the percentages of identity remained very high (>96%), therefore the risk of completely disregarding a specific gene family was low with any of the tools. Additionally, the predicted phenotypes were largely coherent, as most gene variants contained the expected antimicrobial agents within the respective field (with the limitations described in section 4.1). For a minority of cases, **the reported variant did have an impact on the *in silico* predicted phenotype**. The two instances of differences in variant identification across strains were:

- **CARD-RGI** identified *aac(6')-Ib-cr* as *aac(6')-Ib10* and only listed aminoglycosides as the expected phenotype. The gene was identified by ResFinder as *aac(6')-Ib-cr* and by AMRFinderPlus as *aac(6')-Ib-cr5*, both associated with resistance towards fluoroquinolones and aminoglycosides. This problem happened for all data types, file input formats and submission methods. The limitation was observed for all strains that harboured the gene and for which CARD-RGI did not miss it (n=4).
- **ResFinder** identified *aac(3)-IIa* variant (reference gene accession CP023555) across strains. AMRFinderPlus and CARD-RGI reported *aac(3)-IIe*

(reference protein accession WP_000557452.1, with respective reference assembly NZ_CP183773.1:c38337-37477). BLASTn (megablast) revealed 100% identity and coverage between the two reference sequences. ResFinder and CARD-RGI listed the antimicrobials gentamicin and tobramycin. However, AMRFinderPlus only listed gentamicin, which is a limitation of the database (section 4.1) and not related to the specific gene variant identified by the tool. This limitation was observed for all strains that harboured this specific variant of the gene (n=4) and for all data types, file input formats and submission methods. Other limitations were observed for other *aac(3)-II* variants in ResFinder, as described above (missing one *aac(3)-IIg*) and in section 4.3 (inconsistent *aac(3)-IId* variant reporting across data types and file formats).

Notably, there were **no problems detected with AMRFinderPlus**, except those directly associated with its database (section 4.1), and a minor inconsistency for related to data type (section 4.3).

There were **six strains with problems independent from specific tools**, however those problems influenced the outputs to different extents:

- For strain 53_S27, a partial *bla*$_{DHA}$ was clearly flagged by AMRFinderPlus. CARD-RGI and ResFinder did not provide a clear flag. ResFinder analysis with fastq files did not report the partial gene. This was likely a true partial and nonfunctional gene.
- For strain ABIsac_ColiR, a *bla*$_{OXA-23}$ was reported by ResFinder with 99.9% of both identity and coverage. However the gene was flagged by AMRFinderPlus as a partial gene with only 78.4% coverage, and it was not reported at all by CARD-RGI. Raw data were not available. It was conservatively assumed that the gene was indeed present in the genome, and that the older sequencing technologies might have introduced artefacts.
- In strain EURGen-2024-02 it was not possible to confidently identify the *aac(6')-Ib* variant present in the genome. This was because the contigs harbouring the fragments (or the multiple copies) of the gene(s) were very short, leading to inconsistency in copy number reported between tools/data types, and leading to different reported variants (due to low coverage).
- In three other strains, ARGs were truncated at the end of the contigs therefore AMRFinderPlus and ResFinder reported different variants. CARD-RGI did not report any gene. Two of these cases were *aac(6')-Ib* in strains 6717008 and Kpn-3-M28196, and raw data were not available to further investigate the gene variants. The other case was *qnrS* in strain KR6077, and raw data showed that the variant was *qnrS1*.

Annex 4 presents all the inconsistencies in results observed for the different tools, as well as the discrepancies due to databases, different data types, input file formats and submission methods (sections 4.1 and 4.3).

The complete output from the analyses with the three tools are available as Supplementary Material in ScienceData: https://sciencedata.dk/shared/2963696a552f6084b776f60fadc4dc3c.

An important consideration is the **complexity of the output provided by each tool**, which affects how the results can be interpreted and reported consistently

across NRLs:

- AMRFinderPlus produced the most structured output, typically assigning a single hierarchical call per locus together with explicit method and annotation fields. Those fields help distinguish genuine determinants from assembly artefacts or truncated genes. It explicitly flags translated frameshifts and premature stop codons (INTERNAL_STOP), and distinguishes between genes truncated at the end of an assembly contig (PARTIAL_CONTIG_END) from genuine partial genes (PARTIAL).
- CARD-RGI required the user to interpret hit categories (Perfect, Strict, Loose) and the "nudge" behaviour. In generally it produced the longest output with the highest number of genes, which may complicate the interpretation if users are not familiar with the different genetic determinants listed in the results.
- ResFinder produced the most concise output, with a simple structure that is easier to interpret by users without a bioinformatics background. It has the advantage of producing an *in-silico* antibiogram for the species included in its curated phenotype table. However, it was also the tool with the lowest number of genetic determinants in its databases, and could produce very convoluted outputs particularly for LR fastq data, where several variants of the same gene were reported for a single locus (section 4.3). That complexity could increase the risk of inconsistent reporting for users who are not aware of the limitation.

Conclusions

AMRFinderPlus contains the most extensive database, especially for PMs, providing broad coverage across all four target species. Moreover, it produces a more focused output than CARD-RGI. It includes details that are relevant for quality control of the assemblies and of the output itself, such as INTERNAL_STOP and PARTIAL_CONTIG_END flags. However, it is currently CLI only, which restricts the use to NRLs with local bioinformatics capacity. The “Subclass” field, intended to list individual antimicrobials, is incomplete and inconsistent across the database (section 4.1).

CARD-RGI had the advantage of allowing CLI and web-based submissions. It also presented one of the richest ontologies linking each gene to drug class and resistance mechanism. However, the database has significant gaps for the genetic determinants relevant to this report, especially PMs, although it offered coverage for all four species. The tool also reports a large number of additional genes beyond the target catalogue, which increases the burden of interpretation for non-expert users. Importantly, CARD-RGI did not report truncated genes located at the end of the contigs, even though those are assembly artefacts and the genes may remain functional. Finally, there were some specific limitations detected during the testing, for example the problematic reporting of *gyrA* and *parC* PMs as if assigned to *E. coli*, missing multiple aminoglycoside resistance genes, and problems with variant detection for *aac(6')-Ib-cr*.

ResFinder output is structured and straightforward to interpret, and it is the only of the three tools that can produce an *in-silico* antibiogram. Additionally, it allows for CLI and web-based submissions. The main limitation was the absence of a curated PM database for CRAb and CRPa. Other limitations were the one case when it did not report *aac(3)-IIg*, and the inconsistent reporting of variants when compared

with the other tools (especially for *aac(3)-II*).

4.3. Impact of data type, input file format and submission method

The impact of data type (SR and LR) was evaluated for all tools, using the 12 EURGen-RefLabCap strains with paired SR and LR data. The effect of input file format (fasta and fastq) was evaluated for ResFinder only, using the same 12 strains and 2 additional strains that contained both fasta and fastq files. The impact of submission method (CLI and webtool) was evaluated for CARD-RGI and ResFinder, for all strains.

The data type and input file format affected the results. Many of the issues were related to the submission of LR fastq files, and therefore were only observed for ResFinder since the other two tools do not accept fastq files. However, inconsistencies were also seen with CARD-RGI, especially for LR data. To a lesser extent, **submission method also had an impact** on the results. These issues are summarized below and fully described in **Annex 4**.

Overall, the **problems observed for ResFinder** were the following:

- The webtool only accepts files with maximum size of 1 GB, which prevented the submission of three LR fastq files which exceeded that limit [EURGen-2024-02, EURGen-2024-03 and EURGen-2024-04]. The files could be analysed by CLI. The limit imposed by the webtool may constitute a problem for users working with LR sequencing technology, which historically produces larger raw data files when compared with SR technologies.
- LR fastq files submitted via CLI produced very poor output when compared to the respective webtool submission, the assembled genome analysed with both submission methods, and all the SR results. Specifically, ResFinder struggled to select a specific gene variant to assign to each locus. Instead, several different variants of the same gene were provided in the output, with no indication that all the genes could correspond to the same locus in the genome. This was observed for all 12 strains where LR data was available for testing. This complex output may be problematic for users that are not aware of this limitation, bringing difficulties related to reporting protocols and data comparability.
- The multiple reporting of genes for the same locus was also observed in other cases, to a much smaller extent. Specifically, it happened with the webtool submission of LR fastq file for one strain [EURGen-2022-01], and with SR fastq files (both submission methods) for two strains [EURGen-2024-02, KR6077].
- LR fastq files submitted via CLI also created unexpected reports of genes or PMs that were not found with other data types, file formats nor submission methods, and not reported by any other tool: *bla*$_{\text{NDM-1}}$, *rmtC and aac(6')-Ib11* [EURGen-2022-03]*, parC* E84K [EURGen-2023-02], *bla*$_{\text{NDM-1}}$ and *parC* E84K [EURGen-2024-01], *qnr* and *bla*$_{\text{OXA-181}}$ [EURGen-2024-03], and *aac(6')-Ib11* [EURGen-2024-04].
- Certain outputs were affected by the input file format, with reported hits that were not present for other file formats nor in other tools, therefore likely incorrect. In strain EURGen-2022-02 *aac(6')-Ib11* was reported with fastq files but not fasta files, and in strain EURGen-2024-02 *qepA* was reported with fasta files but not fastq. In strain EURGen-2023-02 there was a minor

inconsistency with SR fastq files reporting *aac(3)-IIa* instead of *aac(3)-IId.*

CARD-RGI also had problems that seemed to be associated with specific data type or submission method:

- It struggled with LR sequencing data in a few instances and it failed to report certain genes that were found in the results obtained for SR data and in other tools: *rmtC* [EURGen-2022-02], *bla*$_{\text{NDM-1}}$ [EURGen-2023-01], *aac(6')-Il* [EURGen-2023-04].
- For the strain EURGen-2023-04, *aac(3)-Id* was also absent from both LR outputs and moreover from the SR webtool output. It was only reported for SR data by the CLI.
- For two strains, the LR sequence outputs included *aac(6')-Ib* genes but those were missing from the SR outputs [EURGen-2023-02, EURGen-2024-04].

There was **one minor difference detected with AMRFinderPlus**, where *bla*$_{\text{NDM-5}}$ was identified with SR data, but LR data could not assign a specific NDM variant. The only comparison performed with the tool was between SR and LR fasta files submitted via CLI, as there is no webtool and it does not accept other file formats.

Additional considerations, not necessarily related with the specific tools tested in this report, are as follows:

- Results obtained with fastq files generally lack the information of 'position in contig', due to the structure of raw data files. Results obtained with fasta files show the position in the contig for the detected genes, which can be an advantage for surveillance purposes, as it may suggest if different genes are located in the chromosome or in plasmids. There are further bioinformatics tools that can perform this evaluation.
- Differences between SR and LR results are strongly affected by the base-calling and assembly versions available for each sequencing technology. Such comparisons may benefit from re-evaluation as LR base-calling accuracy continues to improve.

Conclusions

Overall, during the testing for this report, SR data in the format of fasta files had the best performance. These were readily accepted by all tools through all submission methods, and produced less inconsistencies than other data types or file formats.

LR data had limitations across tools. Generally, in CARD-RGI, some of the expected ARGs were not reported. Generally, in ResFinder, these data created reports of genes or PMs that were not present in the genome.

In ResFinder (the only tool that accepted such files) LR data in fastq format created hits not observed for other data types or formats.

There were minimal differences between CLI and web-based submissions, and most of those were related to the point above (multiple hits for the same loci when submitting LR fastq files to ResFinder via CLI, when compared to the respective webtool output).

5. EURL-PH-AMR recommendations

Table 4 summarises the EURL-PH-AMR proposal for the minimum catalogue of **ARGs and chromosomal PMs to be monitored in Europe for surveillance of CCRE, CRAb and CRPa**. The association between specific ARGs and chromosomal PMs with their target antimicrobials is presented for surveillance purposes only. WGS-based results should not be used to infer phenotypic AMR profiles.

Table 4. *EURL-PH-AMR proposal for the minimum catalogue of genes and chromosomal point mutations to be monitored in Europe for surveillance of antimicrobial resistance in CCRE, CRAb and CRPa*

ARGs and PMs	**Target antimicrobials [a]**	**Notes and references**
Ambler class B β-lactamases	IPM, MEM, IMR, MEV, CZT, CZA	- *bla*$_{NDM}$ and its variants - *bla*$_{VIM}$, and its variants - *bla*$_{IMP}$ and its variants [9,18,19]
Ambler class A β-lactamases	IPM, MEM, CZT	- *bla*$_{KPC}$ and its variants (some may present reduced carbapenemase activity) - Specific variants of *bla*$_{GES}$ (e.g. *bla*$_{GES-4}$, *bla*$_{GES-5}$, *bla*$_{GES-14}$) [9,18–20]
Ambler class D β-lactamases	IPM, MEM, IMR, MEV, AZA, CZA, CZT	Specific oxacillinases present carbapenemase activity [18,19,21]: - *bla*$_{OXA-48-like}$ (e.g. *bla*$_{OXA-48}$, *bla*$_{OXA-162}$, *bla*$_{OXA-181}$, *bla*$_{OXA-204}$, *bla*$_{OXA-232}$, *bla*$_{OXA-244}$) [1] - *bla*$_{OXA-655}$ (but not other *bla*$_{OXA-10-like}$) [1] - *bla*$_{OXA-198}$ (or other *bla*$_{OXA-198-like}$) [2] - *bla*$_{OXA-24/40-like}$ (e.g. *bla*$_{OXA-24/40}$, *bla*$_{OXA-25}$, *bla*$_{OXA-26}$) [3] - *bla*$_{OXA-23-like}$ (e.g. *bla*$_{OXA-23}$, *bla*$_{OXA-27}$, *bla*$_{OXA-49}$) [4] - *bla*$_{OXA-58}$ (or other *bla*$_{OXA-58-like}$) [4] They are transferable between species but more frequently described in certain hosts: [1] Enterobacterales and *P. aeruginosa* [22–24] [2] *P. aeruginosa* [25] [3] *P. aeruginosa* and *A. baumannii* [26,27] [4] *A. baumannii* [27] Phenotype depends on variant. OXA-48-like enzymes may be inhibited by CZA and AZA, therefore phenotypic resistance towards those antimicrobials is not expected. *bla*$_{OXA-50}$ is intrinsic in *P. aeruginosa* and *bla*$_{OXA-51}$ is intrinsic in *A. baumannii*. By itself, the presence of those genes is not associated with acquired resistance phenotypes.
Ambler class C β-lactamases	CZT	*bla*$_{CMY}$ and its variants [19,21]. Besides the association with resistance towards CZT, AmpC β-lactamases increase the effects of PBP mutations on β-lactam resistance.
PM in *ftsI*	AZA, CZA, CZT, FDC	*ftsI* encodes penicillin-binding protein 3 (PBP3). Phenotype depends on specific PMs. The effect is increased by the association with acquired β-lactamases [28–39]. These PMs contribute to overall reduction of susceptibility towards other β-lactams.

ARGs and PMs	Target antimicrobials [a]	Notes and references
mcr	COL	E.g. *mcr-1* to *mcr-10* and their variants [40]. *mcr-9* is not associated with phenotypic resistance [41].
PM in *pmrA*, *pmrB, phoP, phoQ, mgrB*	COL	Phenotype depends on specific PMs [42–44]. PMs in *mgrB* are relevant in *K. pneumoniae*. PMs in *phoP* and *phoQ* are relevant in *K. pneumoniae* and *P. aeruginosa*.
qnr	CIP, LVX, MXF	E.g. *qnrA*, *qnrB*, *qnrS, qnrVC* and their variants [45].
qep	CIP, LVX, MXF	E.g. *qepA* and its variants [46].
PM in *gyrA, gyrB, parC, parE*	CIP, LVX, MXF	Phenotype depends on specific PMs [45,47–55]. PMs in *gyrB* and *parE* are relevant in *E. coli* and *P. aeruginosa*.
arm	AMK, GEN, TOB	E.g. *armA* and its variants [56,57].
rmt	AMK, GEN, TOB	E.g. *rmtA* to *rmtH* and their variants [56,57].
ant(2'')-Ia	GEN, TOB	[56–60]
aph(3')-VI	AMK	And its variants [56,57,60,61].
aac(3)-I	GEN	And its variants [56,57,59,60].
aac(3)-II	GEN, TOB	And its variants [56,57,59,60].
aac(6')-I	AMK, TOB	And its variants [56,57,59,60].
aac(6')-Ib-cr	CIP, LVX, MXF, AMK, TOB	And its variants [45,56,57].
aac(6')-II	GEN, TOB	And its variants [56,57,60].

ARG: Antimicrobial resistance gene; PM: Point mutation; PBP: penicillin-binding protein.
[a] AMK: Amikacin; AZA: Aztreonam-avibactam; CIP: Ciprofloxacin; COL: Colistin; CZA: Ceftazidime-avibactam; CZT: Ceftolozane-tazobactam; FDC: Cefiderocol; GEN: Gentamicin; IMR: Imipenem-relebactam; IPM: Imipenem; LVX: Levofloxacin; MEM: Meropenem; MEV: Meropenem-vaborbactam; MFX: Moxifloxacin; TOB: Tobramycin.

The bioinformatics tools and associated databases recommended for the analysis of each bacterial pathogen depend on local capacity at each NRL. The EURL-PH-AMR proposes a stratified approach:

1. In the NRLs with CLI capacity, it is **recommended to use AMRFinderPlus** for all EURGen-Net pathogens, for identification of acquired ARGs and chromosomal PMs.
2. In the NRLs without CLI capacity, it is recommended to use:
    i. **CARD-RGI webtool**, for all EURGen-Net pathogens, for identification of acquired ARGs and chromosomal PMs.
    ii. **Complement the analyses with ResFinder webtool**, to ensure that no *aac(6')-Ib* variants were misreported in the CARD-RGI output, to confirm that no other aminoglycoside resistance genes or any truncated ARGs were absent from CARD-RGI output, and to confirm the chromosomal PMs in *K. pneumoniae*.

NRLs are encouraged to **use fasta files**, as overall there were less inconsistencies

detected for fasta files when compared with fastq files. Additionally, fasta files can be submitted across all bioinformatics tools, and generally produce outputs that are easier to interpret.

NRLs are encouraged to continue to **use the sequencing technologies implemented in their settings**, taking into consideration the **limitations of LR fastq files**. These were the files that produced the worst results. In general, the problems were also related to the fastq file format, and mostly mitigated by the use of LR fasta files.

Several additional **operational recommendations** are made to minimise tool-specific artefacts and support inter-laboratory data comparability:

- Confirm species identification before AMR analysis. If the species is defined incorrectly for detection of AMR, the wrong reference content will be interrogated and have an impact on the output.
- Register the names and versions for each tool and database, as well as the date of the analysis. This information is essential for traceability, retrospective re-analysis and for inter-laboratory comparability.
- Hits below the identity or coverage thresholds, or that are truncated at contig boundaries, should not necessarily be filtered out. Truncations at the end of the contig can indicate assembly artefacts; these are explicitly flagged by AMRFinderPlus as PARTIAL_CONTIG_END. Moreover, mutations resulting in internal stop codons are flagged as INTERNAL_STOP. NRLs are recommended to make use of these annotations to distinguish probable assembly artefacts from genuine partial or inactivated genes. When two tools disagree on specific gene variant for the same isolate, the AMRFinderPlus call is recommended as the primary option for allele naming.

Good quality of WGS data is a pre-requisite for successful bioinformatics analyses and to ensure accuracy and comparability of results. Considerations regarding DNA extraction, DNA quality assessment protocols, sequencing strategies, and WGS data quality control are outside of the scope of this report. However, the EURL-PH-AMR will publish other guidance documents elaborating further on those issues (i.e. protocols on genotypic testing methods).

6. Future perspectives

This is the first version of the EURL-PH-AMR report on bioinformatic tools and reference functional gene databases for detection of AMR for surveillance purposes. The EURL-PH-AMR plans to create a second version in 2028, and a third version in 2030.

This report focuses on the bioinformatics tools and databases most frequently reported by European NRLs in recent past activities. Additionally, it aims at providing recommendations that can be readily applied for the analysis of the majority of pathogens under surveillance within the network. However, these do not correspond to all the bioinformatics tools that could potentially be useful within the EURL-PH-AMR network. Therefore, future updates to this report may include other bioinformatics tools of relevance. Examples are Pathogenwatch[11], Enterobase[12], Kleborate[13] and ArdAP[14]. These tools target particular species (Enterobacterales and *P. aeruginosa*) and are validated and curated by experts, which likely improves the reporting and interpretation of results.

Moreover, the antimicrobials listed in this report do not entirely cover all agents included in phenotypic surveillance activities, within EURL-PH-AMR focus, nor all that are routinely tested at the NRLs [8,62,63]. Therefore, future updates to this report may expand the list of antimicrobials. These may include, for example, fosfomycin and tigecycline for *E. coli*, and trimethoprim-sulfamethoxazole for Enterobacterales and *A. baumannii*.

Likewise, the AMR genes and chromosomal PMs included in this report are not a fully comprehensive list of all genetic mechanisms that may affect the respective antimicrobials. They represent mechanisms that are of epidemiological importance within Europe, or otherwise of interest to the NRLs, while simultaneously being well-described in scientific literature with strong support for their impact on bacterial phenotypes. Thus, future versions of this report may include other relevant genes of mutations, such as other carbapenemases (e.g. bla_{IMI} [64]), β-lactamases associated with decrease in cefiderocol susceptibility (e.g. bla_{PER} [65,66]), and chromosomal PMs in porins (e.g. *ompK* [67,68], *oprD* [69,70]).

Finally, other species of emerging epidemiological importance in Europe might be considered, especially Enterobacterales such as *Enterobacter* spp. [71].

[11] https://pathogen.watch/
[12] https://enterobase.warwick.ac.uk/
[13] https://github.com/klebgenomics/Kleborate
[14] https://github.com/dsarov/ARDaP

Annex 1. Bioinformatics tools in place in European NRLs, 2022-2024

Table A1. *Methods reported by the participants for detection of genetic determinants of antimicrobial resistance in the 2022 [12], 2023 [13] and 2024 [14] EURGen-RefLabCap genomic proficiency tests*

Year	**2022**		**2023**		**2024**	
LC	**Type**	**Software and databases**	**Type**	**Software and databases**	**Type**	**Software and databases**
001	Local and WB	ResFinder, AMRFinderPlus	WB [a]	ResFinder, AMRFinderPlus	Local and WB	ResFinder, AMRFinderPlus
002	Local	AMRFinderPlus, staramr, ARIBA with French Reference Center DB	WB	ResFinder	Local	Resfinder, AMRFinderPlus, ABRicate
003	WB	ResFinder	WB	ResFinder	-	-
004	Local and WB	AMRFinderPlus, ResFinder, ABRIcate	Local	AMRFinderPlus, ResFinder, CARD-RGI	Local and WB	ResFinder, abriTAMR
005	WB	ResFinder	-	-	-	-
007	Local and WB	AMRFinderPlus, ResFinder	-	-	-	-
008	NR	ResFinder	NR	ResFinder	-	
009	Local and WB	ResFinder, in-house script	Local	In-house script with ResFinder DB	WB	ResFinder
010	WB	ResFinder	NR	ResFinder	Local	ResFinder, AMRFinderPlus
011	Local and WB	ResFinder, SRST2 with CARD DB	Local and WB	ResFinder, SRST2 with CARD and ARG-ANNOT DBs	Local and WB	ResFinder, SRST2 with CARD and ARG-ANNOT DBs
012	Local	In-house script and DB	Local	In-house script with ResFinder, CARD and AMRFinderPlus DBs	-	-
014	Local and WB	ResFinder, Ridom SeqSphere+	Local and WB	ResFinder, Ridom SeqSphere+	Local	Ridom SeqSphere+
015	WB	ResFinder	WB	ResFinder	WB	ResFinder
016	WB	ResFinder, CARD-RGI	WB	ResFinder, CARD-RGI	Local	ResFinder, AMRFinderPlus
017	WB [a]	BioNumerics with E. coli plugin	NR	BioNumerics with E. coli plugin	Local	Ridom SeqSphere+

Year	2022		2023		2024	
LC	**Type**	**Software and databases**	**Type**	**Software and databases**	**Type**	**Software and databases**
018	WB	ResFinder	WB	ResFinder	WB	ResFinder, AMRFinderPlus through Galaxy
019	Local	ResFinder, CARD-RGI	Local	ResFinder, AMRFinderPlus, CARD-RGI	Local	ResFinder, AMRFinderPlus, CARD-RGI
020	Local	In-house script with AMRFinderPlus DB	NR	Ridom SeqSphere+	NR	Ridom SeqSphere+
021	WB	ResFinder	NR	Ridom SeqSphere+ with additional CARD and ResFinder DBs	Local and WB	ResFinder, AMRFinderPlus
022	WB	ResFinder	WB	ResFinder	-	-
023	Local	AMRFinderPlus	Local	AMRFinderPlus, Kleborate	Local	AMRFinderPlus, Kleborate
024	WB	ResFinder	WB	ResFinder	-	-
025	-	-	-	-	Local	AMRFinderPlus, ABRicate
026	WB	ResFinder	WB	ResFinder	WB	ResFinder
027	Local and WB	ResFinder, Ridom SeqSphere+	NR	ResFinder, Ridom SeqSphere+	Local and WB [a]	Ridom SeqSphere+
028	WB	ResFinder	WB	ResFinder	-	-
029	Local and WB	ResFinder, KmerResistance, ABRIcate	Local	Ridom SeqSphere+	Local and WB	ResFinder, AMRFinderPlus
030	NR	ResFinder, MGE	Local	ResFinder, AMRFinderPlus	Local	ResFinder, AMRFinderPlus
031	Local and WB	ResFinder, Ridom SeqSphere+, ARIBA with ResFinder, CARD and MEGAres DBs	Local and WB	ResFinder, ARIBA with CARD DB	WB	ResFinder, staramr through Galaxy
032	Local	In-house script with public DBs	-	-	-	-
033	-	-	NR	ResFinder, AMRFinderPlus	Local	abriTAMR
034	-	-	Local	ResFinder	Local	ResFinder
035	-	-	NR	ResFinder, CARD-RGI	-	-
036	-	-	-	-	Local	AMRFinderPlus

DB: database; LC: Laboratory code; WB: web-based; NR: not reported.

[a] Potentially incomplete or inaccurate, based on the reported software and databases.

Summary of the tools frequently reported by the participants:

- ABRicate: Local tool with bundle of databases including ResFinder, AMRFinderPlus and CARD (https://github.com/tseemann/abricate)
- abriTAMR: Local tool with AMRFinderPlus database (https://github.com/MDU-PHL/abritamr)
- AMRFinderPlus: Local tool with own database (NCBI) (https://github.com/ncbi/amr)
- ARIBA: Local tool with self-selected databases (https://github.com/sanger-pathogens/ariba)
- BioNumerics: Discontinued commercial local tool with self-selected databases (https://bio.tools/bionumerics)
- CARD-RGI: Local and web-based tool (RGI) with own database (CARD) (https://github.com/arpcard/rgi; https://card.mcmaster.ca/analyze/rgi)
- Galaxy: Web-based interface for creation of pipelines with diverse bioinformatics tools (https://usegalaxy.org/)
- Kleborate: Local tool for *Klebsiella pneumoniae* (with CARD database) and *Escherichia coli* (with AMRFinderPlus database) (https://github.com/klebgenomics/Kleborate)
- ResFinder: Local and web-based tool with own database (https://github.com/genomicepidemiology/resfinder; https://genepi.food.dtu.dk/resfinder)
- Ridom SeqSphere+: Commercial local tool with AMRFinderPlus database (https://www.ridom.de/)
- SRST2: Local tool with self-selected databases (https://github.com/katholt/srst2)
- Staramr: Local tool with ResFinder database (https://github.com/phac-nml/staramr)

Annex 2. Genomic dataset used for testing the bioinformatics tools

Table A2. *Genomic dataset used for testing the bioinformatics tools including the type of data files and the sample accession*

Strains and references	**Species**	**Relevant genetic determinants of AMR [a]**	**Data types and file formats**	**ENA sample accession**
EURGen-2022-01 [12]	Eco	*bla*$_{NDM-1}$	SR and LR, fastq and fasta	ERS29507654
EURGen-2022-02 [12]	Kpn	*bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *rmtC*, *aac(3)-IIe*, *aac(6')-Ib-cr, gyrA S83I*, *parC* S80I	SR and LR, fastq and fasta	ERS29507655
EURGen-2022-03 [12]	Kpn	*bla*$_{KPC-3}$, *qnrB1*, *aac(3)-IIe*, *aac(6')-Ib-cr*, *gyrA* S83I, *parC* S80I, *mgrB*::IS*1*	SR and LR, fastq and fasta	ERS29507656
EURGen-2022-04 [12]	Eco	*bla*$_{OXA-436}$ (48-like), *qnrA1*, *mcr-9.1*, *aac(3)-IIg*, *aac(6')-IIc, gyrA* S83L	SR and LR, fastq and fasta	ERS29507657
EURGen-2023-01 [13]	Aba	*bla*$_{NDM-1}$, *bla*$_{OXA-23}$, *armA, aph(3')-VI, gyrA* S81L, *parC* S84L, *parC* V104I, *parC* D105E, *ftsI* A515V	SR and LR, fastq and fasta	ERS18629982
EURGen-2023-02 [13]	Eco	*bla*$_{NDM-5}$, *bla*$_{OXA-181}$, *bla*$_{CMY-2}$, *qnrS1*, *aac(3)-IId*, *aac(6')-Ib-cr*, *gyrA* S83L, *gyrA* D87N, *parC* S80I, *parE* S458A, *ftsI* N337NYRIN, *pmrB* Y358N	SR and LR, fastq and fasta	ERS18629983
EURGen-2023-03 [13]	Kpn	*qnrS1, mgrB*::IS*1*	SR and LR, fastq and fasta	ERS18629984
EURGen-2023-04 [13]	Pae	*bla*$_{VIM-2}$, *aac(3)-Id*, *aac(6')-Il*, *gyrA* T83I, *parC* S87L	SR and LR, fastq and fasta	ERS18629985
EURGen-2024-01 [14]	Eco	*mcr-1.1, aac(3)-IIe, gyrA* S83L, *gyrA* D87Y, *parC* S80I, *parE* S458A	SR and LR, fastq and fasta	ERS21378605
EURGen-2024-02 [14]	Pae	*bla*$_{NDM-1}$, *bla*$_{IMP-62}$, *qnrVC1*, *aph(3')-VIa*, *aac(6')-Ib* [b], (*qepA* [b]), *gyrA* T83I, *parC* S87L	SR and LR, fastq and fasta	ERS21378606
EURGen-2024-03 [14]	Aba	*bla*$_{OXA-23}$, *ant(2'')-Ia*, *gyrA* S81L, *parC* S84L, *parC* V104I, *parC* D105E	SR and LR, fastq and fasta	ERS21378607
EURGen-2024-04 [14]	Kpn	*bla*$_{NDM-5}$, *bla*$_{OXA-181}$, *qnrS1*, *rmtB*, *aac(6')-Ib-cr*, *gyrA* D87N, *gyrA* S83F, *parC* E84K, *mgrB* W20R	SR and LR, fastq and fasta	ERS21378608
KR6077 [72]	Kpn	*bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *armA*, *qnrS1* [c], *ant(2")-Ia, aph(3')-VI, aac(6')-Ib-cr, gyrA* S83I, *parC* S80I, *pmrB* R256G, *pmrB* M240T, *phoP* E82K, *phoP* E57K	SR fastq and fasta [f]	SAMN41015955
53_S27 [73]	Kpn	*bla*$_{NDM-1}$, *armA*, *aac(6')-Ib-cr, aac(3)-IIe, gyrA* D87G, *gyrA* S83Y, *parC* S80I, *parC* N304S	SR fastq and fasta [f]	SAMN04011455
Kpn-3-M28196 [33]	Kpn	*bla*$_{NDM-5}$, *bla*$_{KPC-2}$, *rmtB*, *aac(3)-IId, aac(6')-Ib* [d], *gyrA* S83I, *parC* S80I, *pmrB* R256G, *ftsI* A413V	SR fasta	SAMN39296144

Strains and references	Species	Relevant genetic determinants of AMR [a]	Data types and file formats	ENA sample accession
ABIsac_ColiR [74]	Aba	*bla*$_{OXA-23}$, *aph(3')-VIa, ant(2'')-Ia*, *gyrA* S81L, *parC* S84L, *parC* V104I, *parC* D105E, *pmrA* E8D	SR fasta	ERS380136
6717008 [75]	Pae	*bla*$_{GES-5}$, a*nt(2")-Ia, aac(6')-Ib* [e], *gyrA* T83I, *parC* S87L	SR fasta	SAMN43389277
LiP9 [37,76]	Pae	*gyrA* D87Y, *ftsI* A454V, *ftsI* R504C	SR fasta	SAMN07424162

Aba: A. baumannii; AMR: Antimicrobial resistance; Eco: E. coli; ENA: European Nucleotide Archive; Kpn: K. pneumoniae; LR: Long-read; Pae: P. aeruginosa; SR: Short-read

[a] AMR genes and chromosomal point mutations relevant in the context of this report. Created by consensus of the genetic determinants detected for the preparation of this report and of the information described in the references. The full output from all bioinformatics tools is available as supplementary material.

[b] The specific copy number and/or variant(s) of the aac(6')-Ib gene(s) harboured by the strain could not be determined for this report, due to truncated contigs in the assembled data. The original reference proposes several variants as alternatives, specifically aac(6')-Ib, aac(6')-Ib-cr, ant(3'')-Ii-aac(6')-Iid. Similarly, for this report, there was not sufficient evidence that qepA was present in the genome, although it was proposed in the original reference.

[c] qnrS is truncated at the end of the contig. The specific variant could be determined with results for raw data.

[d] aac(6')-Ib is truncated at the end of the contig. The specific variant could not be determined.

[e] The original reference proposes that both aac(6')-Ib-cr and aac(6')-Ib-Hangzhou are present in the genome, however the results obtained for this report show that only one copy of an aac(6')-Ib is present in the assembly, in one locus, truncated at the end of the contig. The specific variant could not be determined.

[f] Fasta file produced at DTU Food from the publicly available fastq files.

Annex 3. Description of chromosomal point mutations in the databases

Table A3. *Chromosomal PMs identified in each of the databases and associated limitations*

Genes	**AMRFinderPlus**	**CARD-RGI**	**ResFinder** (Eco and Kpn [a])
ftsI	**Eco**: A498T, G363S, G363V, I336IKYRI, I336IPYRI, L350F, N337NYRIN, R335RVPYR, S365Y **Kpn**:L367Q, V355G **Aba**:A515V, T526S **Pae**:A419G, A454V, A539T, F507L, F533L, G63S, H394R, L434V, L461V, N427L, N427S, P527S, Q458R, Q475R, R504C, R504L, S368L, S538L, V471G, V523A, V523M, Y367C	**Eco**: None. PBP3 present within acquired genes but no specific PMs listed. **Kpn**: L367Q **Aba**: None **Pae**: None	**Eco**: None **Kpn**: None
pmrA	**Eco**: None **Kpn**: G53C, G53S, S42N **Aba**: E8D, M12I **Pae**: L157Q	**Eco**: None **Kpn**: None **Aba**: None **Pae**: None. *basR* present within the acquired genes but no specific SNPs listed.	**Eco**: R81S, S39I **Kpn**: None
pmrB	**Eco**: A159P, A159V, C84R, C84Y, E121K, E121Q, E166K, G206D, L10P, L10R, L14Q, L14R, P94A, P94L, P94Q, P94S, RPISLR6del, T147A, T156M, T92P, V161G **Kpn**: ALNQLV129del, D313N, L17Q, L82R, R14del, S208N, S85R, T140P, T157P, Y209del **Aba**: A226T, A227V, E301G, G315D, I164F, L168K, P170L, P233S, P233T, Q270P, R231L, R263L, S17R, T232I, T235I **Pae**: A247T, A248T, A248V, A54V, D47del, G188D, H340R, L14P, L167P, L17Q, L243Q, M292T, P456S, R259H, S257D, S257N, T253M, T343A, V15I, V281I, V9A	**Eco**: None **Kpn**: None **Aba**: None **Pae**: None. *basS* present within the acquired genes but no specific SNPs listed.	**Eco**: V161G **Kpn**: None
phoP (Kpn and Pae)	**Kpn**: D191Y, I201N, V3F **Pae**: A110V, N188H, N188Y, R117L	**Kpn**: D191Y **Pae**: A110V, R117L	**Kpn**: None
phoQ (Kpn and Pae)	**Kpn**: A20P, A20S, D266DD, D438N, G385S, L26P, L348Q, L396Q, L96P, R16C, S174N, S56R, V24G **Pae**: D433Ter, I421Ter, K123Q, LG364del, N104I, V152Ter, V260G	**Kpn**: V24G **Pae**: K123Q, V260G	**Kpn**: None

mgrB (Kpn)	**Kpn**: A14S, C28F, C28Y, C39Ter, C39Y, D31N, F35I, G37S, I45T, L17R, L24H, M27K, Q30Ter, V32G, W20R, W47R**.** No insertion sequences included.	**Kpn**: None. *mgrB* present within the acquired genes but no specific SNPs listed. Resistance mechanism was 'resistance by absence'.	**Kpn**: None
gyrA	**Eco**: A51V, A67S, A84P, A84V, A196E, D82G, D87G, D87H, D87N, D87V, D87Y, G81C, G81D, H80P, N57K, Q106H, Q106R, S83A, S83F, S83I, S83L, S83V, S83W **Kpn**: D87A, D87G, D87H, D87N, D87Y, S83F, S83I, S83L, S83T, S83Y, T83I **Aba**: G79C, S81L **Pae**: D72G, D87G, D87H, D87N, D87Y, G81C, G81D, H80R, Q106L, T83A, T83I, Y86N	**Eco**: A67S, A84P, D87G, D87H, D87N, D87V, D87Y, G81C, S83I, S83L, S83W, Q106H **Kpn**: None **Aba**: G79C, S81L **Pae**: D87H, D87G, D87N, H80R, T83I	**Eco**: A51V, A67S, A84P, A84V, A196E, D82G, D87A, D87G, D87H, D87N, D87V, D87Y, G81C, G81D, S83A, S83L, S83V, S83W, Q106H, Q106R **Kpn**: D87A, D87G, D87N, S83L, S83F
gyrB (Eco and Pae)	**Eco**: D426N, K447E**.** Other PMs listed associated with aminocoumarin resistance. **Pae**: A372L, A372V, E468D, E468H, E483D, I424L, L464I, S466F, S466Y	**Eco**: None. A few PMs listed associated with aminocoumarin resistance. **Pae**: None	**Eco**: D426N, K447E
parC	**Eco**: A56T, A85T, A108T, A108V, D111H, E84A, E84G, E84K, E84R, E84V, F60I, F60L, G78C, G78D, G78K, S129P, S57T, S80F, S80I, S80L, S80R, S80W, S80Y **Kpn**: E84K, S80I, S80I, S80R **Aba**: E88K, S84F, S84L, S84W **Pae**: S87L, S87W	**Eco**: E84G, S80I **Kpn**: None **Aba**: D105E, S84L, V104I **Pae**: S80L	**Eco**: A56T, A108V, A108T, E84G, E84K, E84V, E84A, E84R, F60I, F60K, G78D, G78C, S57T, S80I, S80L, S80R, S80W **Kpn**: E84K, S80I
parE (Eco and Pae)	**Eco**: D475E, D476N, E460D, E460K, G423R, I355T, I444F, I464F, I470M, I529L, L416F, L445H, P439S, S458A, S458L, S458T **Pae**: A473V, E459D, E459L, M437I, S457G, S457T	**Eco**: D476N **Pae**: A473V, M437I	**Eco**: D476N, E460D, G423R, I355T, I444F, I464F, I470M, I529L, L416F, L445H, P439S, S458T, S458A

Orange text: point mutations fully absent from the database, for that species.
Aba: A. baumannii; Eco: E. coli; Kpn: K. pneumoniae; Pae: P. aeruginosa; PM: point mutations; SNP: single nucleotide polymorphism.
[a] ResFinder does not contain PMs databases for A. baumannii nor P. aeruginosa.

Annex 4. Limitations in the results obtained with the bioinformatics tools

Table A4. *Limitations in results obtained with the bioinformatics tools, with the different data types, different input file formats and different submission methods*

Strains, species and data	Limitations in results
EURGen-2022-01 (Eco) (SR+LR, fasta+fastq)	**RF:** Multiple hits for the same locus with LR fastq files (CLI: 19 *bla*$_{NDM}$; webtool: 9 *bla*$_{NDM}$)
EURGen-2022-02 (Kpn) (SR+LR, fasta+fastq)	**RF**: *gyrA* S83I absent from all outputs (the specific PM does not exist in the DB for this species) **RF**: *aac(6')-Ib11* incorrectly reported with fastq (SR CLI and webtool, LR only CLI); <90% identity and coverage for SR data but >90% for LR **CR**: *rmtC* absent from LR outputs (CLI and webtool) **CR**: *gyrA* and *parC* PMs assigned to Eco **CR**: *aac(6')-Ib-cr* identification was inconsistent: *aac(6')-Ib10* in CR, *aac(6')-Ib-cr* in AFP and RF **RF**: *aac(3)-IIa* nomenclature was inconsistent for the same nucleotide sequence (*aac(3)-IIe* in AFP and CR) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 14 *bla*$_{NDM}$, 5 *aac(3)-II*, 3 incorrect *aac(6')-Ib*)
EURGen-2022-03 (Kpn) (SR+LR, fasta+fastq)	**All**: *mgrB*::IS*1* absent from all outputs (the specific PM does not exist in any of the DBs for this species) **RF**: *gyrA* S83I absent from all outputs (the specific PM does not exist in the DB for this species) **CR**: *gyrA* and *parC* PMs assigned to Eco **RF**: *bla*$_{NDM-1}$, *rmtC and aac(6')-Ib11* incorrectly reported with LR fastq (only CLI); >90% identity and coverage **CR**: *aac(6')-Ib-cr* identification was inconsistent: *aac(6')-Ib10* in CR, *aac(6')-Ib-cr* in AFP and RF **RF:** *aac(3)-IIa* nomenclature was inconsistent for the same nucleotide sequence (*aac(3)-IIe* in AFP and CR) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 15 *bla*$_{KPC}$, 5 *aac(3)-II*, 2 *qnrB*, 2 incorrect *aac(6')-Ib*)
EURGen-2022-04 (Eco) (SR+LR, fasta+fastq)	**RF**: *aac(3)-II* absent from all outputs (*aac(3)-IIg* in AFP and CR) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 2 *mcr-9*, specifically the expected *mcr-9.1* and unexpected *mcr-9.3*); potentially influence of multiple copy number, however other genes with multiple copies are not reported more than once for this output
EURGen-2023-01 (Aba) (SR+LR, fasta+fastq)	**RF**: No PM DB for the species **CR**: *ftsI* A515V absent from all outputs (the specific PM does not exist in the DB for this species) **AFP**: *parC* V104I and *parC* D105E absent from all outputs (the specific PMs do not exist in the DB for this species) **CR**: *bla*$_{NDM-1}$ absent from LR outputs (CLI and webtool) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 9 *bla*$_{NDM}$, 8 *bla*$_{OXA-23-like}$)
EURGen-2023-02 (Eco) (SR+LR, fasta+fastq)	**All**: *pmrB* Y358N absent from all outputs (the specific PM does not exist in any of the DBs for this species) **RF**: *ftsI* N337NYRIN absent from all outputs (the specific PM does not exist in the DB for this species). **CR**: *parE* S458A and *ftsI* N337NYRIN absent from all outputs (the specific PMs do not exist in the DB for this species)

Strains, species and data	Limitations in results
	RF: *aac(3)-IId* was reported as *aac(3)-IIa* with SR fastq (CLI and webtool) **RF**: *parC* E84K incorrectly reported with LR fastq (only CLI) **CR**: *aac(6')-Ib-cr* absent from SR outputs (CLI and webtool) **AFP**: *bla*$_{NDM-5}$ was reported as *bla*$_{NDM}$ with LR data **CR**: *aac(6')-Ib-cr* identification was inconsistent: *aac(6')-Ib10* in CR, *aac(6')-Ib-cr* in AFP and RF **RF:** Multiple hits for the same locus with LR fastq files (CLI: 3 *bla*$_{NDM}$, 12 *bla*$_{CMY}$, 2 *qnrS*, 2 *aac(3)-II*)
EURGen-2023-03 (Kpn) (SR+LR, fasta+fastq)	**All**: *mgrB*::IS*1* absent from all outputs (the specific PM does not exist in any of the DBs for this species) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 4 *qnrS*)
EURGen-2023-04 (Pae) (SR+LR, fasta+fastq)	**RF**: No PM DB for the species **CR**: *parC* S87L absent from all outputs (the specific PM does not exist in the DB for this species) **CR**: *aac(3)-Id* absent from SR output (only webtool) and from LR outputs (CLI and webtool) **CR**: *aac(6')-Il* absent from LR outputs (CLI and webtool) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 3 *bla*$_{VIM}$)
EURGen-2024-01 (Eco) (SR+LR, fasta+fastq)	**CR**: *parE* S458A absent from all outputs (the specific PM does not exist in the DB for this species) **RF**: *bla*$_{NDM-1}$ and *parC* E84K incorrectly reported with LR fastq (only CLI); >90% identity and coverage **RF**: *aac(3)-IIa* nomenclature was inconsistent for the same nucleotide sequence (*aac(3)-IIe* in AFP and CR) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 18 *mcr-1*, 3 *aac(3)-II*)
EURGen-2024-02 (Pae) (SR+LR, fasta+fastq)	**RF**: No PM DB for the species **CR**: *parC* S87L absent from all outputs (the specific PM does not exist in the DB for this species) **All**: Unresolved *aac(6')-Ib* copy number and/or variant(s) due to truncated contigs and low coverage **RF**: LR fastq not accepted by webtool due to size **RF**: *qepA* incorrectly reported with fasta (SR and LR, CLI and webtool) with 85.1% identity and 94.2% coverage. Absent from fastq results. Absent from other tools' outputs. **RF:** Multiple hits for the same locus with SR fastq files (CLI and webtool: 2 *aph(3')-VI*) **RF:** Multiple hits for the same locus with LR fastq files (CLI: 17 *bla*$_{NDM}$, 2 *bla*$_{IMP}$, 2 *qnrVC*, 3 *aph(3')-VI*)
EURGen-2024-03 (Aba) (SR+LR, fasta+fastq)	**RF**: No PM DB for the species **CR**: *bla*$_{OXA-23}$ absent from SR output (CLI and webtool) **AFP**: *parC* V104I and *parC* D105E absent from all outputs (the specific PMs do not exist in the DB for this species) **RF**: LR fastq not accepted by webtool due to size **RF**: *qnr* and *bla*$_{OXA-181}$ incorrectly reported with LR fastq (only CLI); >90% identity and coverage **RF:** Multiple hits for the same locus with LR fastq files (CLI: 19 *bla*$_{OXA-23-like}$, 2 incorrect *qnr*)
EURGen-2024-04 (Kpn) (SR+LR, fasta+fastq)	**RF**: *mgrB* W20R absent from all outputs (the specific PM does not exist in the DB for this species) **CR**: *mgrB* W20R and *parC* E84K absent from all outputs (the specific PMs do not exist in the DB for this species) **RF**: LR fastq not accepted by webtool due to size

Strains, species and data	Limitations in results
	CR: *aac(6')-Ib10* absent from SR output (CLI and webtool) **CR**: *gyrA* PMs assigned to *Salmonella* **RF**: *aac(6')-Ib11* incorrectly reported with LR fastq (only CLI); >90% identity and coverage **CR**: *aac(6')-Ib-cr* identification was inconsistent: *aac(6')-Ib10* in CR, *aac(6')-Ib-cr* in AFP and RF **RF:** Multiple hits for the same locus with LR fastq files (CLI: 11 bla_{NDM}, 5 $bla_{OXA-48-like}$, 9 *qnrS*)
KR6077 (Kpn) (SR, fasta+fastq)	**All**: *pmrB* R256G, *pmrB* M240T, *phoP* E82K and *phoP* E57K absent from all outputs (the specific PMs do not exist in any of the DBs for this species) **RF**: *gyrA* S83I absent from all outputs (the specific PM does not exist in the DB for this species) **CR**: *armA, ant(2")-Ia, aac(6')-Ib-cr* and *qnrS* absent from outputs **CR**: *gyrA* and *parC* PMs assigned to Eco **RF:** Multiple hits for the same locus with SR fastq files (CLI and webtool: 2 *ant(2")-Ia*)
53_S27 (Kpn) (SR, fasta+fastq)	**All**: *parC* N304S absent from all outputs (the specific PM does not exist in any of the DBs for this species) **RF**: *gyrA* S83Y absent from all outputs (the specific PM does not exist in the DB for this species) **CR**: *gyrA* S83Y absent from all outputs (the specific PM does not exist in the DB for this species) **All**: Partial bla_{DHA} reported with fasta (all tools, CLI and webtool). Flagged by AFP as partial with 69.9% coverage and 100% identity, reported by RF with 69.8% coverage and 100% identity, reported by CR with 89.2% coverage and 99.6% identity. Absent from RF fastq results. Likely true partial gene. **CR**: *aac(6')-Ib-cr* absent from outputs **CR**: *gyrA* and *parC* PMs assigned to Eco/*Salmonella* **RF**: *aac(3)-IIa* nomenclature was inconsistent for the same nucleotide sequence (*aac(3)-IIe* in AFP and CR)
Kpn-3-M28196 (Kpn) (SR, fasta)	**All**: *pmrB* R256G and *ftsI* A413V absent from all outputs (the specific PMs do not exist in any of the DBs for this species) **RF**: *gyrA* S83I absent from all outputs (the specific PM does not exist in the DB for this species) **CR**: *aac(6')-Ib* absent from outputs **CR**: *gyrA* and *parC* PMs assigned to Eco
ABIsac_ColiR (Aba) (SR, fasta)	**RF**: No PM DB for the species **CR**: *pmrA* E8D absent from all outputs (the specific PM does not exist in the DB for this species) **AFP**: *parC* V104I and *parC* D105E absent from all outputs (the specific PMs do not exist in the DB for this species) **All**: bla_{OXA-23} flagged by AFP as partial as partial with 78.4% coverage and 100% identity, reported by RF with 99.9% both coverage and identity, not reported by CARD-RGI. Potential artefacts from older sequencing technologies. **CR**: *ant(2")-Ia* and bla_{OXA-23} absent from outputs
6717008 (Pae) (SR, fasta)	**RF**: No PM DB for the species **CR**: *parC* S87L absent from all outputs (the specific PM does not exist in the DB for this species) **All**: Unresolved *aac(6')-Ib* variant due to truncated gene at the end of the contig and low coverage **CR**: *aac(6')-Ib* absent from outputs

Strains, species and data	Limitations in results
LiP9 (Pae) (SR, fasta)	**RF**: No PM DB for the species **CR**: *gyrA* D87Y*, ftsI* A454V and *ftsI* R504C absent from all outputs (the specific PMs do not exist in the DB for this species)

Black text: limitation associated with the databases; Orange text: specific limitation associated with the strain or species; Blue text: general limitation observed regardless of strain or species.
Aba: A. baumannii; AFP: AMRFinderPlus; CLI: command-line interface; CR: CARD-RGI; DB: database; Eco: E. coli; Kpn: K. pneumoniae; LR: Long-read; Pae: P. aeruginosa; PM: point mutation; RF: ResFinder; SR: Short-read

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