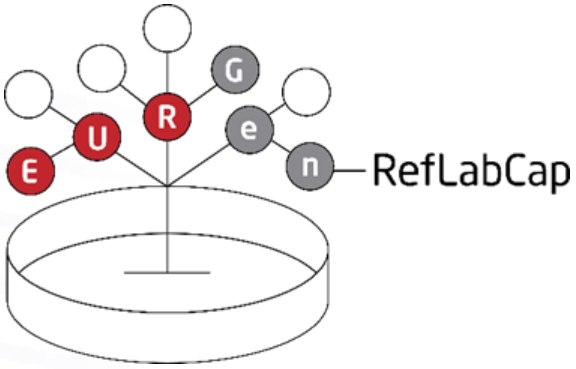
European Commission

EURGen-RefLabCap

Report from the third external quality assessment exercise



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Third EU Health Programme

Report from the third external quality assessment exercise

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EUROPEAN COMMISSION
Directorate-General Health and Food Safety (DG SANTE)
Directorate B — Public health, Cancer and Health security
Unit B2 — Health security
L-2920 Luxembourg
Email : SANTE-CONSULT-B2@ec.europa.eu

European Health and Digital Executive Agency (HaDEA)
HaDEA COV2
Place Rogier, 16
B-1049 BRUXELLES
Belgium
Email : HaDEA-HP-TENDER@ec.europa.eu

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Disclaimer:

For the purposes of this document, this activity is referred to as an External Quality Assessment exercise (EQA). Currently, however, there are no harmonized standard methods for producing and analyzing results through whole-genome sequencing technologies. Therefore, this activity could instead be referred to as a Proficiency Test (PT).

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EXECUTIVE SUMMARY

This report presents the results from the third external quality assessment report (EQA) exercise in EURGen-RefLabCap project, conducted in 2024. The objective of the EQA was to assess the capacity of participating laboratories to produce high quality sequencing data and obtain accurate bioinformatics results using those data.

The EURGen-RefLabCap 2024 EQA included the handling of four live bacterial strains from the species *Escherichia coli*, *Klebsiella pneumoniae*, *A. baumannii* and *P. aeruginosa*, followed by DNA extraction, WGS and bioinformatics analysis. Participants received the live bacterial cultures and also pre-isolated DNA from the same strains and were expected to submit results obtained by sequencing and analysing both sets of material. The submitted results were compared with the expected results and the participants received individual evaluation reports describing their performance in the EQA. The participants were also asked to submit the sequence data that they produced for the EQA and received an evaluation of the quality of those data. Participants unable to perform WGS could instead receive sequence data for analysis. For each type of material, participants were asked to report the multi-locus sequence type (MLST), the plasmid replicon types, the antimicrobial resistance (AMR) genes and/or chromosomal point mutations (PM) mediating AMR, and the associated *in silico* prediction of AMR profiles.

The expected results for the EURGen-RefLabCap 2024 EQA were prepared by performing short-read and long-read sequencing both at the Technical University of Denmark (DTU) and at Statens Serum Institut (SSI) and proceeding with the bioinformatics analysis of the raw and assembled data from both types of sequencing. Consensus results were determined between all datasets and used as the expected results for the EQA.

The invitation for the EURGen-RefLabCap 2024 EQA was sent to the 39 NRLs from 37 countries that participate in the project. Of those, 32 laboratories signed up for the EQA exercise and 31 submitted their results, representing 79.5% of the total laboratories in the project. Of the NRLs that submitted results, 27 laboratories submitted results for all pathogens and the other four laboratories submitted results for only *E. coli* and *K. pneumoniae*. Most laboratories (n=21) analysed both the live bacteria and the pre-isolated DNA. Additionally, six laboratories only analysed either live bacteria (n=4) or DNA (n=2). The remaining four laboratories analysed sequence data produced by the EQA provider.

Most laboratories achieved satisfactory scores in the quality control analysis of the sequence data that they produced for the EQA. Short-read sequence data were submitted by 23 laboratories and more than half (60.9%) achieved scores of 85% or higher for *E. coli* and *K. pneumoniae*, while 75.5% scored above 85% for *A. baumannii* and *P. aeruginosa*. Seven laboratories achieved the maximum score of 100% across all submissions, while another seven achieved 95% or above for all submitted files. The EQA revealed a challenge in the use of long-read sequencing, and those data showed lower yield that prevented reliable coverage across plasmids.

For MLST, 27 participants submitted results, specifically they submitted 193 MLST predictions. Of these, 184 were correct (95.5%). The predictions of ST for *E. coli*, *K. pneumoniae*, and *P. aeruginosa* were in full concordance with the expected results. However, there were nine discordances reported for the *A. baumannii* strain, due to the use of the participants using different MLST scheme for than the one used to prepare expected results.

For plasmid replicons, 31 participants submitted results, specifically they submitted 104 sets of results. The average concordance between submitted and expected replicons was

81.3%. Nearly half of the submitted sets of results (49%, n=51) were fully correct, but 50% (n=53) were missing certain expected replicons. Participants reported unexpected replicon in 16.3% of results. Discordances might be due to different tools or databases reporting different replicons for the same sequence, or due to reporting of several similar replicons.

For AMR genes and mutations, 31 participants submitted results, specifically they submitted 193 sets of results. The average concordance between expected and submitted results was 79.6%. More than 70% of the sets of submitted results (n=132) were incomplete and missing one or more expected genetic AMR determinants, with most missing determinants being chromosomal PMs associated with AMR. This is likely due to the lack of species-specific chromosomal PM databases in some bioinformatics tools. For example, the most frequently missed PMs for *E. coli* were the *glpT* E448K and *uhpT* E350Q conferring resistance to fosfomycin, which are absent in ResFinder but present in AMRFinderPlus and CARD databases. Many laboratories reported the expected PMs but did not follow the instructions described in the 2024 EQA protocol for submission of PMs in the webtool, resulting in PMs not matching accepted formats and being automatically scored as unexpected.

In silico prediction of AMR profiles was conducted alongside detecting genetic determinants mediating AMR. A total of 173 sets of AMR profiles were submitted by 28 laboratories. The average concordance between expected and submitted results was 85.2%. Out of these profiles, 23.7% (n=41) were fully correct, while 64.2% (n=111) were missing antimicrobials that were part of the expected results. These major discrepancies were mainly due to the lack of reporting predicted resistance towards aztreonam (in *E. coli*, *P. aeruginosa* and *K. pneumoniae*), ciprofloxacin (in all four strains) and ceftazidime-avibactam (in *E. coli* and *K. pneumoniae*). These are due to limitations in the bioinformatics databases used to predict the AMR profiles, since these either have errors regarding the absence of aztreonam and ceftazidime-avibactam from AMR genes that are associated with resistance towards those antimicrobials, or they are missing the collections of chromosomal PMs for *A. baumannii* and *P. aeruginosa*.

In general, the discordances between expected and reported results were attributed to variations in bioinformatics tools and databases, as well as the lack of databases for detecting plasmids and genetic AMR determinants for *Acinetobacter* and *Pseudomonas* species. These discrepancies highlight the need for international harmonization of bioinformatics approaches. To increase local capacity in WGS analyses, laboratories should adopt harmonized protocols, align their pre-exiting approaches with harmonized protocols, ensure sufficient knowledge about genetic mechanisms mediating AMR, analyze results critically, and ensure accurate reporting into electronic systems.

The feedback survey for the EURGen-RefLabCap 2024 was completed by 12 laboratories, and the 2024 EQA received an average score of 9 out of 10. Ten participants found the evaluation reports clear and useful, and six participants took corrective actions based on those reports.

1. INTRODUCTION

1.1. Background

The EURGen-RefLabCap project is complementary to the European Centre of Disease Prevention and Control (ECDC) European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net). The project aims at improving capacities of National Reference Laboratories (NRLs) in European countries for identification and for phenotypic and genotypic characterization of carbapenem-resistant *Enterobacterales* (CRE) and colistin-resistant CRE (CCRE), as well as carbapenem- and/or colistin-resistant *Pseudomonas aeruginosa* (C/CRPa) and *Acinetobacter baumannii* complex (C/CRAb). Furthermore, the project aims at strengthening capacities for national surveillance and outbreak investigation of CRE/CCRE, C/CRPa and C/CRAb, and improve the availability and quality of European-level molecular surveillance data. One of the main goals of the EURGen-RefLabCap project is to support modernisation of diagnostic and molecular typing tests using whole-genome sequencing (WGS) analytical methods to achieve those respective aims.

External quality assessment (EQA) exercises are an important tool to assess the capacity of laboratories to follow their own routine procedures and obtain accurate results. This assessment is done by comparing the achieved results with expected results produced by standard methods, and with results obtained by other laboratories. EQAs may also allow for comparing the performance and accuracy of different laboratory protocols and pipelines for analysis of WGS data. This can be possible if the results submitted by participants, for the same type of analyses, are obtained using different methods.

1.2. EQAs in the EURGen-RefLabCap project

Within the EURGen-RefLabCap project, three EQAs were planned (Figure 1) to evaluate and ensure the quality and comparability of the WGS-based data on resistome profiling and high-risk clone identification produced by the NRLs for CRE/CCRE (workstream 1 (WS1) pathogens), and C/CRPa and C/CRAb (workstream 2 (WS2) pathogens). The main objective of the EURGen-RefLabCap EQAs is to assess the laboratories' proficiency regarding WGS and bioinformatics analysis of the relevant pathogens. Results obtained by the participants are compared with the expected results obtained by the Technical University of Denmark (DTU) and Statens Serum Institut (SSI) to assess if WGS-based analysis results are reliable and of consistently good quality. Results from the EQAs will help in planning relevant guidance and training, and potentially encourage laboratories in addressing shortcomings related to their individual results.

WGS data have not yet been properly validated to be used for clinical diagnostic purposes. Some of the analyses included in the EURGen-RefLabCap EQAs have important limitations when considering their applicability in clinical microbiology laboratories, such as the *in silico* prediction of AMR profiles. Thus, the EURGen-RefLabCap EQAs are not an assessment of laboratories' capacity or ability to accurately perform their routine confirmatory, diagnostics or surveillance procedures. Instead, the EQAs aim at comparing bioinformatics approaches used by the NRLs in Europe, to benchmark the performance of those approaches, to identify potential problems or variation between the applied pipelines, and to identify local, national, and European opportunities for quality improvement and harmonization of analysis of WGS data.

The EURGen-RefLabCap EQAs were planned in order of increasing challenge (Figure 1). The first EQA, conducted in 2022, included WS1 pathogens and encompassed analysis of WGS data using the routine bioinformatics approaches applied by the participating laboratories, with the aim of assessing the accuracy and completeness of those approaches. The second EQA, focusing on WS1 and WS2 pathogens, also included DNA sequencing, to furthermore evaluate the capacity for WGS in the individual laboratories and to analyse the quality of locally produced WGS data. The final EQA, again including WS1 and WS2 pathogens, and the focus of this current report, included the handling of live bacterial isolates and DNA extraction, to also assess local capacity for those steps of the sequencing process.

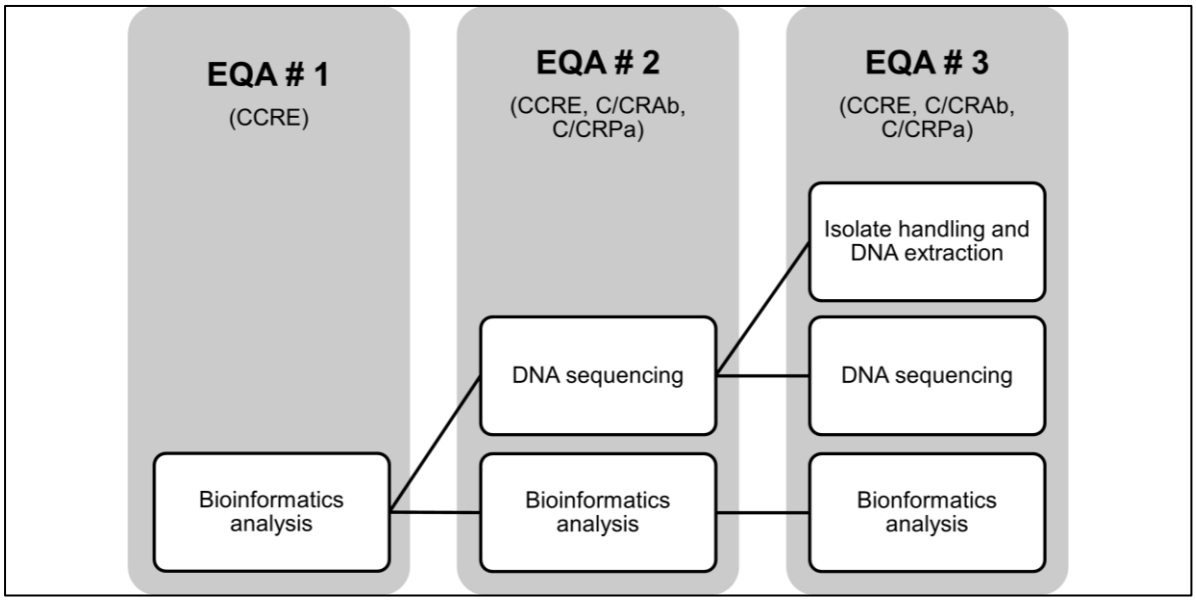


Figure 1. Representation of the three EQAs conducted in the EURGen-RefLabCap project

The third EURGen-RefLabCap EQA included live cultures as well as pure vacuum-dried DNA samples of four strains (one *Escherichia coli*, one *Klebsiella pneumoniae*, one *Acinetobacter baumannii* and one *Pseudomonas aeruginosa* strain). The laboratories nominated for WS1 of the EURGen-RefLabCap project could submit results for the *E. coli* and *K. pneumoniae* strains, and the laboratories nominated for WS2 of the project could submit results for the *A. baumannii* and *P. aeruginosa* strains. Laboratories nominated for both workstreams could submit results for all four strains. The EQA included: i) DNA extraction and sequencing with any desired technology; ii) prediction of sequence type (ST) based on multilocus sequence typing (MLST); iii) detection of plasmid replicon types; iv) detection of genes and chromosomal point mutations (PMs) mediating antimicrobial resistance (AMR), and v) *in silico* prediction of the AMR profiles. All NRLs that participate in the EURGen-RefLabCap project (n=39) were invited to complete the EQA exercise. The number of laboratories that signed up for the EQA exercise was 32 and 31 laboratories submitted their results into the EQA webtool. Of these, four laboratories submitted results for only WS1 pathogens, and 27 laboratories submitted results for all pathogens.

2. EXERCISE DESIGN AND METHODS

2.1. EQA material

The materials in EURGen-RefLabCap 2024 EQA included one *E. coli* (EURGen-2024-01), one *P. aeruginosa* (EURGen-2024-02), one *A. baumannii* (EURGen-2024-03) and one *K. pneumoniae* (EURGen-2024-04) strain. For each strain of the above-mentioned bacterial species, laboratories were expected to handle and process two types of test materials, as described below:

- BACT: Live bacterial cultures (referred to as "BACT") for which DNA extraction and purification, library-preparation, WGS, and *in silico* analyses was expected to be performed. The cultures were provided as swabs in transport media.

- DNA: Pre-isolated DNA (referred to as "DNA") for which library-preparation, WGS, and *in silico* analyses was expected to be performed. Each vial contained a minimum of 125 ng DNA.

When signing up for the analysis of BACT samples for one species, analysis of DNA samples was also expected. If, for any reason, participants were unable to handle live cultures and perform DNA extraction, it was possible to submit results only for DNA samples.

The strains were selected based on their genomic content including ST, plasmid replicons and genetic determinants associated with resistance towards carbapenems, colistin and other antimicrobials of clinical relevance. For each strain, participants received one Eppendorf® tube containing at least 125 ng of vacuum-dried DNA and one swab with live culture. In case participants were unable to sequence the DNA, sequence data ("SEQ") was available upon request: either assembled data files (FASTA files) or raw sequence data files (FASTQ files), produced either with short-read sequencing technologies (Illumina, Inc., San Diego, CA, United States of America) or with long-read sequencing technologies (Oxford Nanopore Technologies (ONT), Inc., Oxford, United Kingdom). The sequences are available under accession number PRJEB79393 at the European Nucleotide Archive.

Table 1. Overview of material available to the participants for the EURGen-RefLabCap 2024 EQA

Strain	Material code	Description
EURGen-2024-01 (*E. coli*)	EURGen-2024-01_BACT	Swabs containing live bacterial culture
	EURGen-2024-01_DNA	Approximately 125 ng of pure, vacuum-dried DNA
	EURGen-2024-01_Illumina.fasta	Assembled reads produced with short-read sequencing
	EURGen-2024-01_Nanopore.fasta	Assembled reads produced with long-read sequencing
	EURGen-2024-01_Illumina_R1.fastq EURGen-2024-01_Illumina_R2.fastq	Raw data files produced with short-read sequencing
	EURGen-2024-01_Nanopore.fastq	Raw data file produced with long-read sequencing
EURGen-2024-02 (*P. aeruginosa*)	EURGen-2024-02_BACT	Swabs containing live bacterial culture
	EURGen-2024-02_DNA	Approximately 125 ng of pure, vacuum-dried DNA
	EURGen-2024-02_Illumina.fasta	Assembled reads produced with short-read sequencing

Strain	Material code	Description
	EURGen-2024-02_Nanopore.fasta	Assembled reads produced with long-read sequencing
	EURGen-2024-02_Illumina_R1.fastq EURGen-2024-02_Illumina_R2.fastq	Raw data files produced with short-read sequencing
	EURGen-2024-02_Nanopore.fastq	Raw data file produced with long-read sequencing
EURGen-2024-03 (*A. baumannii*)	EURGen-2024-03_BACT	Swabs containing live bacterial culture
	EURGen-2024-03_DNA	Approximately 125 ng of pure, vacuum-dried DNA
	EURGen-2024-03_Illumina.fasta	Assembled reads produced with short-read sequencing
	EURGen-2024-03_Nanopore.fasta	Assembled reads produced with long-read sequencing
	EURGen-2024-03_Illumina_R1.fastq EURGen-2024-03_Illumina_R2.fastq	Raw data files produced with short-read sequencing
	EURGen-2024-03_Nanopore.fastq	Raw data file produced with long-read sequencing
EURGen-2024-04 (*K. pneumoniae*)	EURGen-2024-04_BACT	Swabs containing live bacterial culture
	EURGen-2024-04_DNA	Approximately 125 ng of pure, vacuum-dried DNA
	EURGen-2024-04_Illumina.fasta	Assembled reads produced with short-read sequencing
	EURGen-2024-04_Nanopore.fasta	Assembled reads produced with long-read sequencing
	EURGen-2024-04_Illumina_R1.fastq EURGen-2024-04_Illumina_R2.fastq	Raw data files produced with short-read sequencing
	EURGen-2024-04_Nanopore.fastq	Raw data file produced with long-read sequencing

The DNA samples were prepared at The Technical University of Denmark (DTU) with Invitrogen™ Easy-DNA™ gDNA Purification kit (Thermo-Fischer Scientific, Massachusetts, United States of America). The short-read sequencing data were obtained using Illumina NextSeq™ 500. The libraries were prepared with Illumina Nextera™ XT DNA Library Preparation Kit – 96 Samples using Illumina Nextera™ XT DNA library preparation reference guide (Version 06, August 2021). Long-read sequencing data were obtained with ONT GridION™ using R10.4.1 flow cells (FLO-MIN114). The libraries were prepared with ONT Rapid Barcoding Kit 96 V14, (SQK-RBK114.96 using the Rapid sequencing DNA V14 – barcoding protocol (SQK-RBK114.96, VERSION: RBK_9176_V114_REVA_27NOV2022). The quality control of raw short reads was performed using FastQC[1] v0.11.5 and quality trimming was performed using BBDuK2 v36.49[2]. The quality control of raw long-reads was evaluated using Nanoq[3] v0.10.0 and NanoPlot[4] v1.41.6 and trimming was performed using Filtong[5] v0.2.1. The genome assembly from short-reads was performed using SPAdes Genome Assembler[6] v3.11.0, while genome assembly from long-reads was performed with Flye v2.9.1[7].

[1] https://github.com/s-andrews/FastQC
[2] https://github.com/BioInfoTools/BBMap
[3] https://github.com/wdecoster/nanostat
[4] https://github.com/wdecoster/NanoPlot
[5] https://github.com/rrwick/Filtlong
[6] https://github.com/ablab/spades
[7] https://github.com/fenderglass/Flye

2.2. Expected results

The expected bioinformatics analysis results were produced at DTU and Statens Serum Institute (SSI). At DTU, the expected results were produced using a suite of bioinformatics tools and databases including the tools available at the Center for Genomic Epidemiology (CGE), using the short-read and long-read sequencing data files (FASTA and FASTQ):

- STs were predicted with command-line MLST[8] v2.0.9, using PubMLST[9] database version 2023-12-18. All seven alleles determining ST were found from assemblies and had perfect matches (100% identity).

- Plasmid replicons were detected with webtool PlasmidFinder[10] v2.0.1 (2020-07-01), database version 2023-01-18 with minimum thresholds of identity: 90% and coverage: 90%;

- AMR genes and chromosomal mutations conferring AMR were detected with web-based ResFinder[11] v4.4.2 using ResFinder database version 2023-04-12 and PointFinder database version 2023-05-03, with minimum thresholds of identity: 80% and coverage: 60%. Additionally, command-line AMRFinderPlus[12] v3.12.8 with database version 2024-05-02.2 (minimum thresholds of identity: 90% and coverage: 50%) and Resistance Genes Identifier (RGI)[13] with CARD database v3.2.6 (using "Perfect" and "Strict" detection paradigms) were also used to detect AMR genes and mutations.

To generate the expected results at SSI, the DNA from the cultures was extracted on a Roche MagNA Pure 96 automated extraction platform (F. Hoffmann-La Roche Ltd, Basel, Switzerland) using the Roche Viral NA Small Volume DNA Multi-Sample Kit according to the instructions provided by the manufacturer. The DNA was sequenced with both short-read and long-read sequencing technology. The short-read sequencing data at SSI were obtained using Illumina NextSeq™ 550 using the Illumina Nextera™ XT DNA Library Preparation Kit following the Illumina Nextera™ XT DNA library preparation reference guide (Version 06, August 2021) and a v2.5 Mid Output 300-cycle Sequencing Reagent kit to obtain 2x150bp paired-end reads. The long-read sequencing was performed on the ONT GridION™ platform using R10.4.1 flow cells (FLO-MIN114). The libraries were prepared with Rapid Barcoding Kit V14 using the Rapid sequencing DNA V14 barcoding protocol (SQK-RBK114.96, VERSION: RBK_9176_V114_REVA_27NOV2022). The quality control of raw reads, both for short- and long-read data, was performed using Bifrost[14] QC and analysis pipeline. The genome assembly from short-reads was performed using SKESA[15] v2.4.0, while genome assembly from long-reads was performed with Flye[16] v2.9.3 and polished with Medaka[17] v1.11.3. The results regarding STs, plasmid replicons, genes and chromosomal mutations mediating AMR, and prediction of AMR profiles were obtained by using two methods in parallel, including:

[8] https://cge.food.dtu.dk/services/MLST/
[9] https://pubmlst.org/
[10] https://cge.food.dtu.dk/services/PlasmidFinder/
[11] https://cge.food.dtu.dk/services/ResFinder/
[12] https://github.com/ncbi/amr
[13] https://card.mcmaster.ca/analyze/rgi
[14] https://github.com/pmelsted/bifrost
[15] https://github.com/ncbi/SKESA
[16] https://github.com/mikolmogorov/Flye
[17] https://github.com/nanoporetech/medaka

- STs were predicted with Bifrost v2.0.8 (local pipeline) and Pathogenwatch[18] v22.1.0 (web-based) using default parameters (minimum thresholds of identity: 80% and coverage: 80%);

- Identification of plasmid replicons was performed using web-based Inctyper at Pathogenwatch v22.1.3 (default parameters: minimum thresholds of identity: 90% and coverage: not defined) and web-based PlasmidFinder v2.1 (database version 2023-01-18) with default parameters (minimum thresholds of identity: 90% and coverage: 60%);

- AMR genes and chromosomal mutations mediating antimicrobial resistance were detected using AMRFinderPlus v3.12.8 (database v2024-01-31.1 various tools run with default parameters for percentage of identify and coverage, as follows:

- AMR genes and chromosomal mutations conferring AMR were detected with web-based ResFinder v4.5.2 using ResFinder database version 2023-04-12 and PointFinder database version 2023-05-03, with minimum thresholds of identity: 90% and coverage: 60%. Additionally, AMRFinderPlus v3.12.8 with database version 2024-01-31.1 (minimum thresholds of identity: 90% and coverage: 50%) and Pathogenwatch v22.1.3 were also used to detect AMR genes and mutations.

The consensus expected results were produced by critically evaluating the outcome of the methods used by the two institutions and by choosing thresholds of minimum identity 90% and minimum coverage 90% for identification of plasmid replicons, and minimum identity 90% and minimum coverage 60% for identification of AMR determinants. The expected results are summarised in Tables 2, 3 and 4.

Table 2. Expected MLST results for the material included in the EURGen-RefLabCap 2024 EQA

Material	**ST**	**Alleles assigned to each locus**						
EURGen-2024-01 (*E. coli*)*	**457**	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*
		101	88	97	108	26	79	2
EURGen-2024-02 (*P. aeruginosa*)	**644**	*acsA*	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
		28	3	94	13	1	4	10
EURGen-2024-03 (*A. baumannii*)	**1780**	*cpn60*	*gdhB*	*gltA*	*gpi*	*gyrB*	*recA*	*rpoD*
		1	42	1	159	17	12	6
EURGen-2024-04 (*K. pneumoniae*)*	**16**	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*
		2	1	2	1	4	4	4

* For EURGen-2024-01 (*E. coli*), Achtman scheme was used (*E. coli* #1 on the CGE MLST tool). For EURGen-2024-03 (*A. baumannii*), Oxford scheme was used (*A. baumannii* #1 on the CGE MLST tool).

[18] https://pathogen.watch/

Of note, the PlasmidFinder database is designed to detect plasmids in Enterobacterales. Therefore, it is expected that, by using this method, no plasmid replicons are found in species not belonging to Enterobacterales. At present, replicon typing of plasmids in *A. baumannii* and *P. aeruginosa* is not as standardised as in Enterobacterales.

Table 3. Expected plasmid replicon results for the material included in the EURGen-RefLabCap 2024 EQA

Material	**Plasmid replicons**
EURGen-2024-01 (*E. coli*)	**Expected**: ColpEC648, IncFIA, IncFIB(AP001918), IncFII, IncI1-I(Alpha)
EURGen-2024-02 (*P. aeruginosa*)	No plasmid replicon detected
EURGen-2024-03 (*A. baumannii*)	No plasmid replicon detected
EURGen-2024-04 (*K. pneumoniae*)	**Expected:** Col440II, IncFIB(K), IncFII, IncFII(K), IncN4, IncX3
	Expected but non-mandatory*: Col(pHAD28), ColKP3

* Certain plasmid replicons were only detected with one bioinformatics tool or were detected only in one type of sequence data. Therefore, the submission of these replicons by the participants is considered "non-mandatory". This reflects limitations associated with bioinformatics analyses, but it does not mean that the replicons are not present in the strain or that these replicons are less important than others for surveillance purposes.

Table 4. Expected acquired AMR genes and chromosomal PMs mediating AMR included in the EURGen-RefLabCap 2024 EQA

Material	**AMR genes and chromosomal mutations**
EURGen-2024-01 (*E. coli*)	**Expected**: *aac(3)-IIa*[a], *bla*$_{\text{CTX-M-65}}$, *bla*$_{\text{TEM-1}}$[b], *dfrA12, dfrA17, sul1, sul2, sul3, fosA3, mcr-1.1*[c], *glpT* E448K, *gyrA* D87Y, *gyrA* S83L, *parC* S80I, *parE* S458A, *uhpT* E350Q
EURGen-2024-02 (*P. aeruginosa*)	**Expected:** *aph(3')-VI*[d], *aac(6')-Ib*[e], *bla*$_{\text{IMP-62}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{PME-1}}$, *crpP, qnrVC1, gyrA* T83I
	Expected but non-mandatory*: *ant(3'')-Ii-aac(6')-Iid*[f], *aac(6')-Ib-cr*[g], *bla*$_{\text{KBL-1}}$, *qepA*[h], *nalC* G71E, *nalC* S209R, *parC* S87L
EURGen-2024-03 (*A. baumannii*)	**Expected:** *ant(2'')-Ia, bla*$_{\text{OXA-23}}$, *gyrA* S81L, *parC* S84L
	Expected but non-mandatory*: *parC* D105E, *parC* V104I
EURGen-2024-04 (*K. pneumoniae*)	**Expected:** *aac(6')-Ib-cr*[i], *bla*$_{\text{CTX-M-15}}$[j], *bla*$_{\text{NDM-5}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-181}}$, *bla*$_{\text{TEM-1}}$[b], *dfrA12, qnrS1, rmtB*[k], *sul1, gyrA* D87N, *gyrA* S83F, *parC* E84K
	Expected but non-mandatory*: *mgrB* W20R

* Certain AMR genes or PMs were only detected with one bioinformatics tool or were detected only in one type of sequence data. Therefore, the submission of these genes or PMs by the participants is considered "non-mandatory". This reflects limitations associated with bioinformatics analyses, but it does not mean that the genes or PMs are not present in the strain or that these genes or PMs are less important than others for surveillance purposes.

[a] Either *aac(3)-IIa* or *aac(3)-IIe*

[b] Either *bla*$_{\text{TEM-1}}$ or *bla*$_{\text{TEM-1A}}$ or *bla*$_{\text{TEM-1B}}$ or *bla*$_{\text{TEM-1C}}$ or *bla*$_{\text{TEM-1D}}$

[c] Either *mcr1.1* or *mcr-1.26*
[d] Either *aph(3')-VI* or *aph(3')-Via*
[e] Either *aac(6')-Ib* or *aac(6')-Ib-Hangzhou* or *aac(6')-Ib3* or *aac(6')-Ib4* or *aac(6')-Ib9*
[f] Either *ant(3'')-Ii-aac(6')-Iid* or *ant(3'')-Ih/aac(6')-Iid*
[g] Either *aac(6')-Ib-cr* or *aac(6')-Ib-cr5*
[h] Either *qepA* or *qepA1* or *qepA2* or *qepA4*
[i] Either *aac(6')-Ib-cr* or *aac(6')-Ib-cr5* or *aac(6')-Ib-cr6*
[j] Either *bla*$_{CTX-M-15}$ or *bla*$_{CTX-M-101}$
[k] Either *rmtB* or *rmtB1*

Table 5. Expected in silico prediction of AMR profiles for the material included in the EURGen-RefLabCap 2024 EQA

Material	**Associated prediction of AMR profiles**
EURGen-2024-01 (*E. coli*)	**Expected**: Ampicillin, Aztreonam, Cefepime, Cefotaxime, Ceftazidime, Ciprofloxacin, Colistin, Fosfomycin, Gentamicin, Sulfamethoxazole, Tobramycin, Trimethoprim
EURGen-2024-02 (*P. aeruginosa*)	**Expected:** Amikacin, Aztreonam, Cefepime, Ceftazidime, Ceftazidime-avibactam, Ciprofloxacin, Gentamicin, Imipenem, Meropenem, Piperacillin-tazobactam, Tobramycin
EURGen-2024-03 (*A. baumannii*)	**Expected:** Ciprofloxacin, Gentamicin, Imipenem, Meropenem, Tobramycin **Intrinsic*:** Aztreonam, Fosfomycin
EURGen-2024-04 (*K. pneumoniae*)	**Expected:** Amikacin, Amoxicillin-clavulanic acid, Aztreonam, Cefepime, Cefotaxime, Ceftazidime, Ceftazidime-avibactam, Ciprofloxacin, Ertapenem, Gentamicin, Imipenem, Meropenem, Piperacillin-tazobactam, Sulfamethoxazole, Tobramycin, Trimethoprim **Expected non-mandatory:** Colistin[a] **Intrinsic*:** Ampicillin

* Intrinsic resistance (based on EUCAST Expected Phenotypes Version 1.2, January 2023), not part of the expected results
[a] Detection of *mgrB* W20R mutation, and subsequent inclusion of colistin in AMR profile of this strain, were expected results, but not mandatory to report. This reflects limitations associated with bioinformatics analyses, but it does not mean that the PM is not present in the strain, or that the mutation is not associated with decreased susceptibility towards colistin, or that this PM is less important than others for surveillance purposes.

2.3. Distribution and procedure

On 14th February 2024 all laboratories that participate in the EURGen-RefLabCap project (n=39) were contacted by email and invited to participate in the 2024 EQA. The email contained a prenotification letter with a brief description of the exercise and indicated that deadline for signing up was 15th March. In total, 32 laboratories signed up to participate in the 2024 EQA. On 1st May, the test material (live cultures and pre-isolated DNA) was shipped to the laboratories that signed up for the 2024 EQA. On 1st May, all EQA participants received an email with instructions on how to download the sequence data from the online platform ScienceData[19], and were informed that the protocol for the EQA

[19] https://sciencedata.dk

and the test forms showing the questions that they would encounter on the webtool for submission of results were directly accessible via the EURGen-RefLabCap website[20]. In addition, the EQA participants received information on how to upload their sequencing data for the quality control evaluation. On 6th June, participants received an email informing that the webtool for submission of results[21] was open, and that submission could take place until the deadline of 28th June. This email had attached a guideline to create the password for the webtool and a guideline explaining how to access the webtool and submit the results. Until the deadline for submission of results (28th June), 23 out of 31 laboratories had completed the EQA. The laboratories were contacted to inquire on the status of their analyses and/or submission, and the deadline was extended by two weeks (until 15th July). The EQA was formally completed on 15th July, with results from 31 participating laboratories, representing 30 countries.

The webtool for submission of results has been developed and hosted by DTU for the purpose of similar EQAs and future related EQAs. The participants were asked to sequence the DNA using their desired sequencing platform and the routine methods implemented in their laboratory. They were asked to predict or detect: i) the ST; ii) the plasmid replicon types; iii) the AMR genes and/or chromosomal PMs mediating AMR, and iv) the associated *in silico* prediction of AMR profiles. For the latter two types of analyses (iii and iv), only clinically relevant antimicrobials or those relevant for surveillance purposes should have been considered (Table 6).

Table 6. Relevant antimicrobials that should have been considered in the EURGen-RefLabCap 2024 EQA, according to the bacterial species in each workstream

Bacterial species	**Antimicrobials to consider**	**Nr.**
E. coli and *K. pneumoniae* (WS1 pathogens)	Amikacin, amoxicillin-clavulanic acid, ampicillin, aztreonam, cefepime, cefotaxime, ceftazidime, ceftazidime-avibactam, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, meropenem, piperacillin-tazobactam, sulfamethoxazole, tigecycline, tobramycin, trimethoprim	20
A. baumannii and *P. aeruginosa* (WS2 pathogens)	Amikacin, aztreonam, cefepime, ceftazidime, ceftazidime-avibactam, ciprofloxacin, colistin, fosfomycin, gentamicin, imipenem, meropenem, piperacillin-tazobactam, tobramycin	13

Participants could decide to analyse a selection of the test material, for example only material regarding to the *E. coli* strain, and could decide to submit a subset of results, for example only ST and plasmid replicons. Participants were encouraged to use the "EURGen-RefLabCap harmonized common WGS-based genome analysis methods and standard protocols for national surveillance and integrated outbreak investigations" for Enterobacterales and for *A. baumannii* and *P. aeruginosa*[22] but were welcome to use other WGS analytical set-ups. Thus, they were also asked to report method-related details in relation to the analysis performed, including the bioinformatics tools, databases and parameters used for sequence analyses and generation of results.

[20] https://www.eurgen-reflabcap.eu/resources/eqa
[21] https://eurgen-reflabcap-pt.dtu.dk
[22] https://www.eurgen-reflabcap.eu/protocols-and-guidelines

In total, each participant could submit eight sets of results: two sets of results for each of the four strains, the first one obtained by sequencing the DNA samples and performing bioinformatics analysis and the second one obtained by processing the live cultures, extracting their DNA and performing WGS. Participants unable to sequence DNA, and therefore unable to submit any of those sets of results, could submit only one set of results by analysing FASTA or FASTQ files produced at DTU.

On 13th September, all laboratories that submitted results received an email informing that their individual results were available for download from the webtool. As another attachment, each participant that submitted locally generated sequencing data for quality control also received a report with the evaluation of the quality of their sequencing data. This email also contained a link to a feedback survey about the 2024 EQA, with a deadline of 30th September.

2.4. Scoring system in the webtool

2.4.1. Overview of the scoring system for bioinformatics results

In the webtool, the results submitted by the participants were compared to the expected results. The webtool assigned a score "1" in cases of concordance between reported and expected results, and it assigned a score "0" in cases of discordance between reported and expected results (specifically if participants reported plasmid replicons, AMR genes, chromosomal PMs or antimicrobials that were not part of the expected results). Moreover, the webtool assigned a "blank" if the participants missed any genetic determinants, antimicrobials, or replicons that were part of the expected results. Missing an expected result and therefore receiving a "blank" was not always due to participant's mistake. As an example, an expected genetic determinant cannot be detected if the participant used databases that did not include that determinant. Additionally, an expected determinant could also be missed if the participant selected stricter thresholds for identity and coverage compared to the thresholds used to prepare the expected results. Furthermore, the webtool assigned a "blank" if the participants reported any AMR determinants, antimicrobials, or replicons that were not mandatory to be reported. These included AMR determinants for which there was no consensus between the bioinformatics tools while preparing the expected results, as well as antimicrobials for which the species is expected to be intrinsically resistant as specified in the EUCAST list of expected phenotypes[23] (Version 1.2, January 2023). The scoring system was the same as applied in the second EURGen-RefLabCap EQA in 2023. A complete description of the scoring system is provided in Table 7.

Table 7. Scoring system applied to the analyses included in the EURGen-RefLabCap 2024 EQA

Analysis	Submitted result	Score
Prediction of ST	Correct ST	1
	Incorrect ST	0
Detection of plasmid replicons, AMR genes and chromosomal PMs	Genetic determinant correctly identified	1
	Reporting a genetic determinant that was part of the expected results but not mandatory to report	blank
	Missing a genetic determinant	blank
	Reporting an unexpected genetic determinant	0

[23] https://www.eucast.org/expert_rules_and_expected_phenotypes/expected_phenotypes

In-silico AMR profiles	AMR profile correctly reported for the antimicrobial	1
	Reporting an antimicrobial that was part of the expected results but not mandatory to report, or part of intrinsic resistance	blank
	Missing an antimicrobial	blank
	Reporting an AMR profile for an unexpected antimicrobial	0

The maximum possible score that each laboratory could achieve depended on the number of analyses that they performed and for how many strains they performed those analyses. For each type of analysed data, laboratories that performed all analyses for all strains could obtain as a maximum of 100 points. Table 8 shows the scores regarding each strain and type of analysis included in the 2024 EQA.

Table 8 . Maximum possible score for the laboratories participating in the EURGen-RefLabCap 2024 EQA, per strain and per type of analysis

Material and analysis	**EURGen-2024-01 (*E. coli*)**	**EURGen-2024-02 (*P. aeruginosa*)**	**EURGen-2024-03 (*A. baumannii*)**	**EURGen-2024-04 (*K. pneumoniae*)**	**Total**
Prediction of ST	1	1	1	1	4
Detection of plasmid replicons	5	0	0	6	11
Detection of AMR genes and chromosomal PMs	16	8	4	13	41
In silico prediction of AMR profiles	12	11	5	16	44
Total	**34**	**20**	**10**	**36**	**100**

2.5. Evaluation of sequences submitted by participants

In the 2024 EQA, participants were offered to submit the raw sequencing data that they generated using the live culture test material for quality evaluation. For submission of raw sequencing data (FASTQ files), participants were instructed to use the ScienceData platform[24]. Each participant was assigned a laboratory identification code (Lab ID) and provided with a unique link to their individual folder on the ScienceData platform where they could upload their produced FASTQ files. Participants could submit sequences generated by short-read or long-read sequencing technologies and could submit either single-end or paired-end sequencing data. Furthermore, they were asked to include MD5 file hashes to verify file integrity.

All the sequencing data submitted by the participants were analyzed using standard bioinformatics tools. For the quality control (QC), genome assembly was performed using SPAdes[25] v3.15.3 and the submitted sequences were compared with the reference genomes using sequence alignment program Burrows-Wheeler Aligner (BWA-MEM)[26] v0.7.17. The sequence mapping statistics were generated using samtools[27] v1.2.

[24] https://sciencedata.dk/
[25] https://github.com/ablab/spades
[26] https://github.com/lh3/bwa
[27] https://github.com/samtools/samtools

Summary assembly and read quality parameters were then produced with in-house pipelines. For long-read data, reads QC and filtering were done with Nanoq[28] v0.10.0, mapping to reference conducted with minimap2[29] v2.24 and assembled with Flye[30] v2.9.1. For MLST, alleles and sequence types were predicted using the CGE MLST[31] v2.0.

The submitted sequence data was analysed in R[32] v4.3.2. The submitted genomes underwent an initial screening and exclusion step (Figure 2). Specifically, the genomes which deviated more than 10% in assembly size compared to the reference genomes or had less than 95% of assigned cgMLST alleles were excluded from the succeeding statistical analyses.

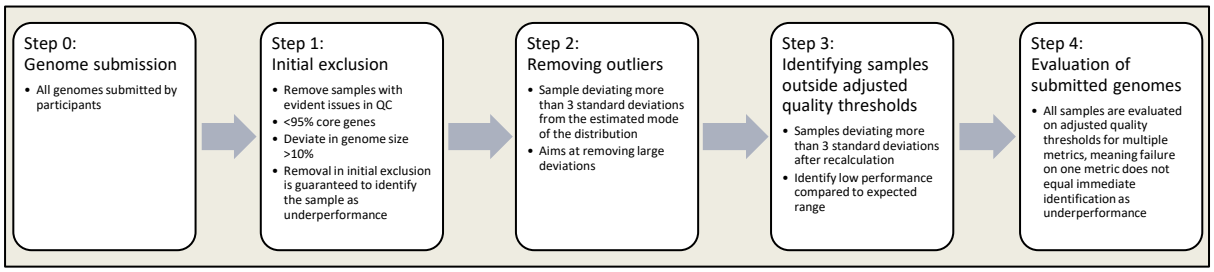


Figure 2. Overview of steps in the data analysis and calculation of thresholds for WGS data evaluation

After excluding the genomes of lower quality, box plots were produced to visualize distribution of QC data. Box plots were prepared with minimum, 1st quantile, median, 3rd quantile and maximum values. Any data points with more than three standard deviations from the estimated mode of the distribution were classified as outliers and were removed from the dataset. After removing the outliers, three standard deviations from the mode of the distribution were recalculated and used as new QC thresholds called adjusted quality thresholds. These adjusted quality thresholds were used for metrics without a predefined cut-off, to identify laboratories not performing comparably to the general quality seen among participants. The adjusted quality thresholds were used for the metrics: size of assembled genome compared to reference, number of contigs above 200 bp, genomic coverage with a minimum depth of 10X, and N50.

A subset of quality parameters was used as indicator for general performance and include widely used routine QC parameters: cgMLST, MLST, average coverage, average Q-score, proportion of reads mapping to reference genome, size of assembled genome compared to reference, number of contigs above 200 bp, genomic coverage with a minimum depth of 10X, N50. An overview of the parameters used for scoring is presented in Table 9. A complete list of the quality parameters evaluated in the 2024 EQA is listed in Appendix 1 and 2.

[28] https://github.com/esteinig/nanoq
[29] https://github.com/lh3/minimap2
[30] https://github.com/fenderglass/Flye
[31] https://cge.food.dtu.dk/services/MLST/
[32] https://www.r-project.org/

Table 9. Overview of the quality control parameters used for scoring and the maximum possible score for each parameter for Illumina short-read sequencing data in the EURGen-RefLabCap 2024 EQA

Group	Metric	Maximum score
Group 1	Q-score Forward reads (R1)	5
	Q-score Reverse Reads (R2)	5
	Depth of coverage: Chromosome	10
	proportion of cgMLST match	15
	MLST	10
	Proportion of reads mapped to reference DNA sequence (%)	5
Group 2	Number of contigs > 200 bp	7.5
	Size of assembled genome per total size of DNA sequence (%)	7.5
Bonus group 2	Pass all group 2 metrics	10
Group 3	Coverage 10x of the reference genome (%)	7.5
	N50	7.5
Bonus group 3	Pass all group 3 metrics	10
Total		100

3. RESULTS AND DISCUSSION

3.1. Participating laboratories and analysed materials

All NRLs that participate in the EURGen-RefLabCap project (n=39) were invited to complete the EQA exercise. The number of laboratories that signed up for the EQA exercise was 32, and 31 laboratories submitted their results (79.5%). These represented 30 of the 37 countries involved in the project (81.1%). This was an improvement from the EQAs in 2022 and 2023 in which laboratories from 27 and 29 countries participated, respectively.

Some laboratories that did not sign up for the 2024 EQA have not yet implemented WGS for the analysis of WS1 or WS2 pathogens.

Of the 31 laboratories that submitted results, four laboratories submitted results for only WS1 pathogens, and 27 laboratories submitted results for all pathogens (Table 10). Most laboratories analysed both BACT and DNA test materials (n=21), but one of these laboratories only analysed BACT sample for the isolate EURGen-2024-02 (*P. aeruginosa*). Additionally, six laboratories only analysed either BACT (n=4) or DNA (n=2). Three laboratories analysed FASTA files produced by the EQA provider, and one laboratory analysed FASTQ files.

Table 10. Materials analysed in the EURGen-RefLabCap 2024 EQA, as reported by participating laboratories (n=31)

Laboratory	**EURGen-2024-01 (*E. coli*)**	**EURGen-2024-02 (P. aeruginosa)**	**EURGen-2024-03 (*A. baumannii*)**	**EURGen-2024-04 (*K. pneumoniae*)**
EURGen-RLC-001	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-002	BACT and DNA	-	-	BACT and DNA
EURGen-RLC-003	FASTA files	FASTA files	FASTA files	FASTA files
EURGen-RLC-004	BACT	BACT	BACT	BACT
EURGen-RLC-005	FASTA files	FASTA files	FASTA files	FASTA files
EURGen-RLC-009	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-010	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-011	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-012	BACT	-	-	BACT
EURGen-RLC-014	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-015	BACT and DNA	BACT	BACT and DNA	BACT and DNA
EURGen-RLC-016	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-017	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-018	DNA	DNA	DNA	DNA
EURGen-RLC-019	BACT and DNA	-	-	BACT and DNA
EURGen-RLC-020	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-021	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-022	DNA	DNA	DNA	DNA
EURGen-RLC-023	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-024	FASTA files	FASTA files	FASTA files	FASTA files
EURGen-RLC-025	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-026	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-027	BACT and DNA	-	-	BACT and DNA
EURGen-RLC-028	FASTQ files	FASTQ files	FASTQ files	FASTQ files

Laboratory	EURGen-2024-01 (*E. coli*)	EURGen-2024-02 (P. aeruginosa)	EURGen-2024-03 (*A. baumannii*)	EURGen-2024-04 (*K. pneumoniae*)
EURGen-RLC-029	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-030	BACT	BACT	BACT	BACT
EURGen-RLC-031	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-032	BACT	BACT	BACT	BACT
EURGen-RLC-033	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-034	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA
EURGen-RLC-036	BACT and DNA	BACT and DNA	BACT and DNA	BACT and DNA

-: Material was not analysed by the laboratory

3.2. Quality control of sequences submitted by participants

In total, 26 participants submitted their locally generated sequencing data for the QC. Most of the participants (n=23) submitted short-read sequencing data generated using Illumina (162 submissions (paired-end data)), while three laboratories submitted long-read sequencing data generated using Nanopore sequencing (19 submissions) (Table 11).

The results of the evaluation of the quality parameters were shared with each participant by email along with the interpretation of results. An overview of Illumina quality parameters is presented in Table 12 for all the participants that submitted Illumina sequencing data for the QC analysis. The total number of submitted genomes included in the analysis was 161 (short-read paired-end sequences), specifically 43 *E. coli*, 38 *P. aeruginosa,* 37 *A. baumannii* and 43 *K. pneumoniae*.

Table 11. Overview of sequencing technology used, and the number of genomes submitted by each participating laboratory in the EURGen-RefLabCap 2024 EQA

Laboratory	Sequencing platform	Defined read length (bp)	No. of files submitted for QC (samples)
EURGen-RLC-001	Illumina iSeq 100	2 x 150	16 (8)
EURGen-RLC-002 (WS1)	ONT GridION	NA	4 (4)
EURGen-RLC-004[a]	ONT MinION	10k	8 (8)
EURGen-RLC-009	Illumina NextSeq 550	2 X 150	16 (8)
EURGen-RLC-010	Illumina NextSeq 2000	2 x 150	16 (8)
EURGen-RLC-011	Illumina MiSeq	2 x 300	16 (8)
EURGen-RLC-012 (WS1)	-	-	4 (2)
EURGen-RLC-014	Illumina NextSeq 1000	2 x 300	16 (8)
EURGen-RLC-015	ONT MinION	10k-100k	7 (7)
EURGen-RLC-016	Illumina NextSeq 550	2 x 150	16 (8)
EURGen-RLC-017	Illumina NextSeq 1000	2 x 300	16 (8)
EURGen-RLC-018	Illumina NextSeq 2000	2 x 150	8 (4)
EURGen-RLC-019 (WS1)	Illumina NextSeq 550	2 x 150	8 (4)
EURGen-RLC-020	Illumina MiSeq	2 x 300	16 (8)
EURGen-RLC-021	Illumina MiniSeq	2 x 300	16 (8)
EURGen-RLC-022[a]	Illumina MiSeq	-	16 (8)
EURGen-RLC-023	Illumina MiSeq	2 x 250	16 (8)
EURGen-RLC-025	Illumina NextSeq 500	2 x 150	16 (8)
EURGen-RLC-026	Illumina MiSeq	2 x 300	16 (8)
EURGen-RLC-027 (WS1)	Illumina iSeq 100	2 x 150	8 (4)
EURGen-RLC-029	Illumina NextSeq 2000	2 x 300	16 (8)
EURGen-RLC-030	Illumina NextSeq 550	2 x 300	8 (4)
EURGen-RLC-031	Illumina NovaSeq 6000	2 x 150	16 (8)
EURGen-RLC-032	Ion S5 prime XL system[b]	NA	NA[c]
EURGen-RLC-033	Illumina MiSeq	2 x 150	16 (8)

Laboratory	Sequencing platform	Defined read length (bp)	No. of files submitted for QC (samples)
EURGen-RLC-034	Illumina NextSeq 550	2 x 150	16 (8)
EURGen-RLC-036	Illumina NextSeq 2000	2 x 100	16 (8)

NA: Not applicable
-: Information not provided by the laboratory. However, laboratory used Illumina sequencing technology.
WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*).
[a] The laboratory submitted sequencing files for both BACT and DNA samples, but only submitted bioinformatics results for either BACT or DNA samples
[b] Thermo Fisher Scientific Inc., Waltham, MA, United States of America.
[c] Sequences using IonTorrent technology were not included in the analysis.

From the initial screening of genome quality, six genomes were excluded from the succeeding statistical analyses for defining QC thresholds due to evident lower quality (EURGen-2024-02 (n=2), EURGen-2024-03 (n=3), EURGen-2024-04 (n=1)). For the evaluation of QC the six genomes were still scored like all other submissions, and the results were reported to the participants (Table 12). Five of these genomes belonged to one laboratory.

Overall, most laboratories performed satisfactorily. Of the 23 laboratories that submitted Illumina sequences for QC analysis, more than half of the laboratories (n=14, 60.9%) achieved scores of 85% or higher of their maximum possible score. The participants achieved scores which correspond to 33.4% to 100% of their maximum possible score. On the species level, 19 of 23 (82.6%) laboratories achieved above 85% of the possible scores for *E. coli* (EURGen-2024-01) and *K. pneumoniae* (EURGen-2024-04). For *A. baumannii* (EURGen-2024-02) and P. *aeruginosa* (EURGen-2024-03), 15 out of 20 (75%) laboratories scored above 85%. Two laboratories scored low across all submitted genomes. Of these, one scored a total of 33.4% and the other scored 53.8% of their maximum possible scores. Seven laboratories achieved the maximum score of 100% across all submissions and another seven achieved 95% or above for all submitted files (Table 12).

Table 12. Maximum possible scores and scores obtained by each participant in the EURGen-RefLabCap 2024 EQA, for each Illumina sequence file submitted, and in total

	Live culture (BACT)				Purified DNA (DNA)				Max.		
Laboratory	EURGen-2024-01	EURGen-2024-02	EURGen-2024-03	EURGen-2024-04	EURGen-2024-01	EURGen-2024-02	EURGen-2024-03	EURGen-2024-04	**Possible score**	**Obtained score**	**Score (%)**
EURGen-RLC-001	100	100	100	100	100	100	100	100	800	800	100
EURGen-RLC-009	97.5	67.5	100	90	97.5	50	80	97.5	800	680	85.0
EURGen-RLC-010	100	100	100	100	100	100	100	100	800	800	100
EURGen-RLC-011	100	100	100	100	100	97.5	97.5	97.5	800	792.5	99.1
EURGen-RLC-012 (WS1)	100	NA	NA	100	NA	NA	NA	NA	200	200	100
EURGen-RLC-014	95	100	100	97.5	100	100	100	100	800	792.5	99.1
EURGen-RLC-016	100	97.5	100	100	100	97.5	100	97.5	800	792.5	99.1
EURGen-RLC-017	100	95	100	100	100	95	NA	100	700	690	98.6
EURGen-RLC-018	NA	NA	NA	NA	100	100	100	100	400	400	100
EURGen-RLC-019 (WS1)	100	NA	NA	100	100	NA	NA	100	400	400	100
EURGen-RLC-020	100	75	55	100	100	90	82.5	100	800	702.5	87.8
EURGen-RLC-021	100	97.5	100	100	100	97.5	100	100	800	795	99.4
EURGen-RLC-022*	45	20	30	45	47.5	20	30	30	800	267.5	33.4
EURGen-RLC-023	100	100	95	100	80	47.5	100	95	800	717.5	89.7
EURGen-RLC-025	100	97.5	100	97.5	100	97.5	100	97.5	800	790	98.8
EURGen-RLC-026	100	100	100	100	100	100	100	100	800	800	100
EURGen-RLC-027 (WS1)	62.5	NA	NA	60	47.5	NA	NA	45	400	215	53.8
EURGen-RLC-029	100	100	100	100	100	95	100	100	800	795	99.4
EURGen-RLC-030	97.5	100	100	80	NA	NA	NA	NA	400	377.5	94.4
EURGen-RLC-031*	100	100	67.5	100	100	100	100	100	800	767.5	95.9
EURGen-RLC-033	92.5	100	100	100	72.5	72.5	100	72.5	800	710	88.8
EURGen-RLC-034	100	100	80	100	100	100	100	100	800	780	97.5
EURGen-RLC-036	100	100	100	100	100	100	100	100	800	800	100.
Average	**95.0**	**92.6**	**92.1**	**87.4**	**90.9**	**93.9**	**94.1**	**92.0**	**NA**	**NA**	**NA**

NA: Not applicable
WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*).
* One or more submissions from these laboratories were excluded from the statistical analyses after the initial screening.

Overall, submitted genomes (n=162, BACT: n=82, DNA: n=80) were within the minimum cut-off values for most submissions (n=142, BACT: n=74, DNA: n=68). Nineteen submitted genomes were outside adjusted quality thresholds, 11 of which were also identified as outliers (Figure 3 and 5). Six genomes failed the initial screening criteria and were excluded from analysis. One genome could not be assembled and was excluded from analysis.

The average phred scores (Q-score) of the submitted raw reads were evaluated. For the forward reads, the average Q-scores for all the sequences for all the test strains were above the preferred cut-off value (>Q30). For reverse reads, all except two genomes were above preferred cut-off for the average Q-score. The two genomes were from EURGen-2024-02 (*P. aeruginosa*) and both submitted by the participant EURGen-RLC-020. These had an average Q-scores above the minimum cut-off value (>Q25), thus no sequence data from any participants were identified as unsatisfactory due to Q-score (Figure 3A and 3B).

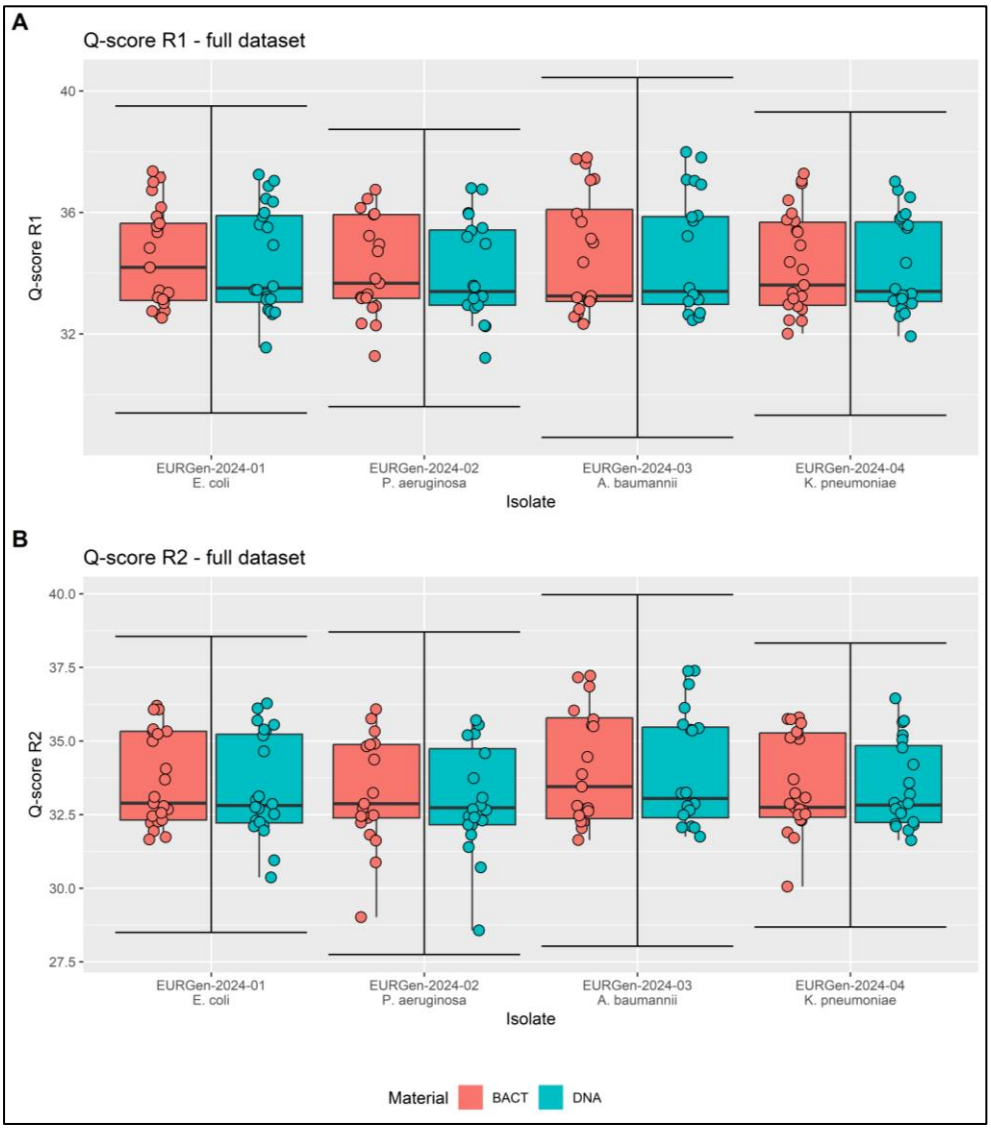


Figure 3. Box plots of average phred score (Q-score) of the Illumina raw reads submitted by the participants for evaluation (n=155 (*E. coli* (n=43), *P. aeruginosa* (n=36), *A. baumannii* (n=34), *K. pneumoniae* (n=42)) in the EURGen-RefLabCap 2024 EQA. Whiskers show the recalculated three standard deviations (referred to as adjusted quality threshold). The red colour shows live culture (BACT) and blue colour shows purified DNA (DNA) genomes. A) Boxplot of average Q-score for forward reads (R1). B) Boxplot of average Q-score for reverse reads (R2).

Contigs less than 200 bp are expected to be non-informative and likely due to the artefacts or residual contaminants from the library preparation step. These contigs are routinely excluded from analysis. The number of contigs above 200 bp should be as low as possible in an assembly, and a large number of short contigs indicates a problem with the raw sequence data, leading to poor assembly. For the analysis, the adjusted quality threshold value of contigs above 200 bp was set at three standard deviations above the median. In all strains, at least one submitted genomes was identified as an outlier. 12 genomes (BACT: n=5, DNA: n=7) were outside the adjusted quality, presented in Figure 4A. These were submitted by six participants, of which four had more than one genome identified outside the adjusted quality threshold.

For the genomic coverage of 10X of the reference genomes, minimum cut-off value was set at three standard deviations below the mean. A coverage depth of 10X at one base position means that 10 sequenced reads cover that base, while the genomic coverage of 10X of the genome represents the percentage of the entire genomic DNA which has at least 10X coverage depth. Overall, most laboratories (n=18) have a genomic coverage above the adjusted quality threshold. In all strains, at least one genome was outside the adjusted quality threshold, EURGen-2024-01 (*E. coli*) (n=3), EURGen-2024-02 (*P. aeruginosa*) (n=2), EURGen-2024-03 (*A. baumannii*) (n=1) and EURGen-2024-04 (*K. pneumoniae*) (n=4), presented in Figure 4B. Most were from purified DNA (BACT: n=4, DNA: n=6). The adjusted quality threshold was in general high, with EURGen-2024-02, EURGen-2024-03 and EURGen-2024-04 having approximately 96% coverage as the lower threshold.

The proportion of reads that align directly to the closed reference genomes was also evaluated for the 23 laboratories that submitted the short-read sequencing data. This metric indicates the amount of possible contamination and non-sense reads in the datasets. The minimum cut-off value was defined as >80%, and preferred cut-off value was set at 90%. Overall, most participants performed well. Three genomes from two participants (EURGen-RLC-009 and EURGen-RLC-014) did not meet the lower threshold of 80% of reads that mapped to the reference genome. Of these, one genome was from EURGen-2024-01 (*E. coli*) and two from EURGen-2024-02 (*P. aeruginosa*).

For comparing the size of assembled genomes with the reference genomes, proportion of assembly size compared to the reference genome was evaluated. The assembly size should be close to 100%, but can deviate due to genomic complexity, such as repeated sequences. The minimum cut-off values were defined as 10% below the expected size, but a preferred threshold of three standard deviations from the mean was also used. The preferred lower threshold was above 98% for all strains, ranging from the lowest 98.11% in EURGen-2024-02 (*P. aeruginosa*) to the highest 98.95% in EURGen-2024-04 (*K. pneumoniae*). Only one genome from BACT sample of EURGen-2024-03 (*A. baumannii*) deviated by more than 10% from the expected genome size, showing clear signs of contamination. Most participants (n=22) in the evaluation of the submitted sequence data passed the cut-off value, see Figure 4C.

For N50 the adjusted quality threshold was calculated as three standard deviations below the median of the log-transformed dataset. Seven genomes from four participants were below the adjusted quality threshold, presented in Figure 4D. The lower thresholds for N50 ranged from 35,245 in EURGen-2024-03 (*A. baumannii*) to 66,549 in EURGen-2024-04 (*K. pneumoniae*).

A total of five laboratories were identified across uncorrelated critical QC parameters (Group 2: Number of contigs >200bp and size of assembly compared to reference, Group 3: N50 and coverage of minimum 10x depth), indicating serious issues in the sequencing runs. These were EURGen-RLC-009, EURGen-RLC-020, EURGen-RLC-022, EURGen-RLC-023 and EURGen-RLC-027. The genomes that fail across uncorrelated QC metrics are regarded as a major error.

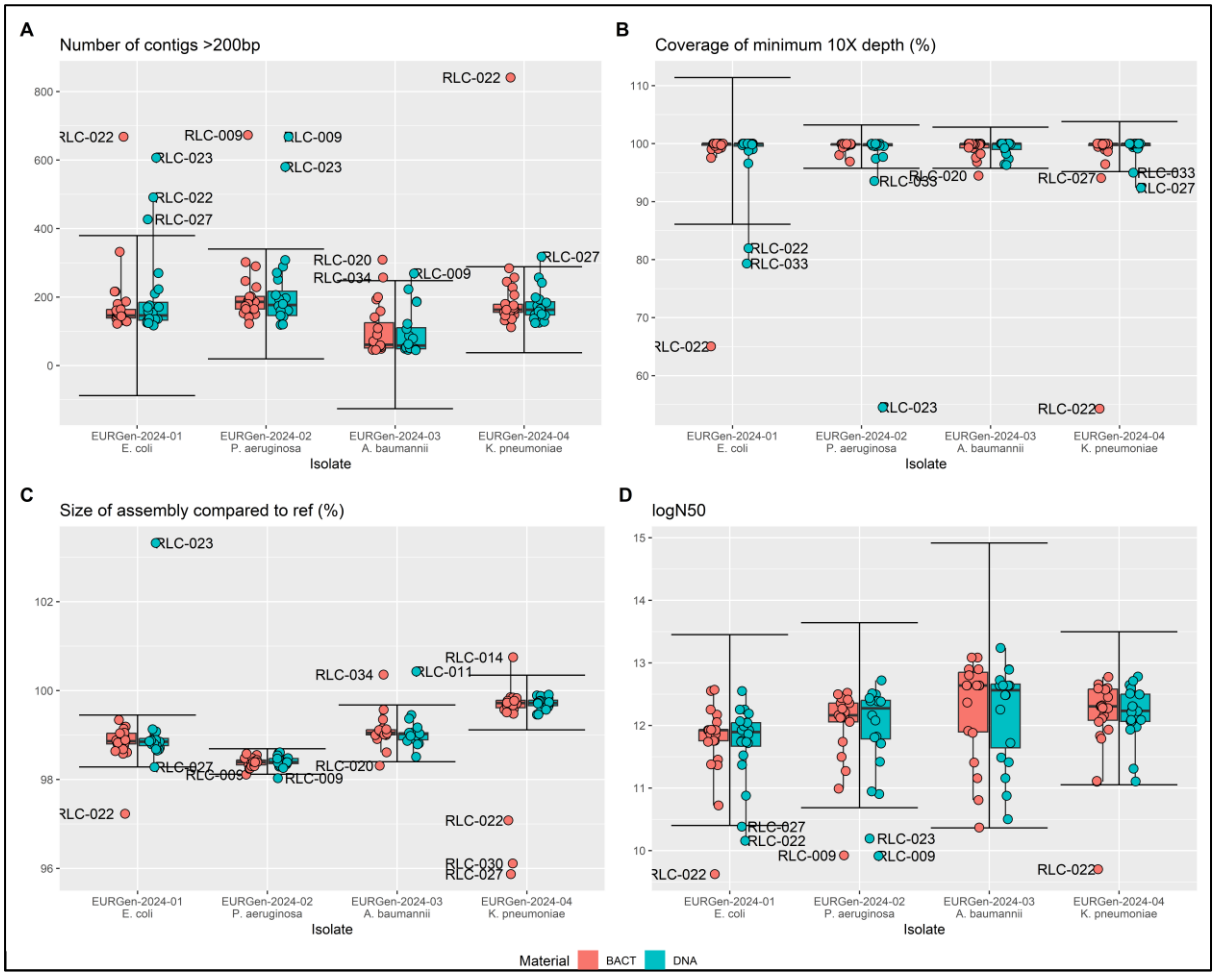


Figure 4. Box plots of some of the QC metrics for the evaluation of Illumina sequences submitted by the participants (n=23) for the EURGen-RefLabCap 2024 EQA. Whiskers show the thresholds for identification of underperformance (referred to as adjusted quality threshold). The dataset used for calculating the adjusted quality threshold removes samples which were identified as outliers in any metric. A) Boxplot of total number of contigs of the assembled genomes for each test strain. B) Boxplot of genomic coverage of at least 10X depth of the genome (%). C) Boxplot of the size of the assembly compared to the size of the reference genome (%). D) Boxplot of the log-transformed N50s.

Long-read sequences (n=19) from three laboratories were evaluated likewise. The QC definitions for ONT sequences are poorly defined as the technology is under continuous development, and the low number of submissions does not allow for statistical inter-laboratory comparison. Based on previous experiences with ONT sequencing and expert recommendations, submissions were evaluated on an individual basis. Overall, the main problem with the long-read sequences was lack of sequencing yield.

Most of the submitted Illumina sequences showed sufficient quality for the type of analysis performed. Overall, quality issues were evenly found in all the bacterial species. The

sequences from one laboratory were significantly below the quality thresholds and achieved a low score of 33.4% of their maximum possible score. Consequently, this lack of quality considerably affected the laboratory's ability to satisfactorily perform bioinformatic analysis and achieved an overall score of 37.5% of the maximum possible score for the bioinformatics analyses (Table 13). This highlights the importance of ensuring good quality sequencing data to accurately detect the genetic AMR determinants and predict the associated AMR profiles. The sequences from another laboratory also chieved relatively low-quality score (53.8%). However, the laboratory achieved above 90% of their maximum possible score for the bioinformatics analyses (Table 13), suggesting that the quality of the sequences was sufficient for the bioinformatics analysis included in the 2024 EQA. A further explanation of the issues observed during the QC of the sequences were provided to individual laboratories to help improve the quality of their sequencing procedures.

For the long-read sequences, the main challenge seen in this EQA to sequencing using ONT was the sequencing yield. In some cases, coverage was only low in BACT genomes or for plasmids, indicating DNA extraction as a particular challenge. Because of the low yield, achieving a reliable coverage across plasmids is not always possible, as all participants had lower coverage on plasmids, and not sufficient to achieve 20X coverage of all plasmids across multiple samples. As plasmids are frequently involved in dissemination of AMR genes, this is regarded as a major concern for the use of these sequences for AMR surveillance.

3.3. Overall scores and evaluation of submitted results

Most laboratories (n=28) submitted results for all four types of analysis included in this EQA, and three laboratories did not submit results for *in silico* prediction of AMR profiles for any strain (Table 13). All participating laboratories correctly identified the species of the four strains included in the 2024 EQA. For all analyses evaluated in this EQA, the concordance between submitted and expected results varied between 35.7% and 99.0% (Figure 5, Table 13). For BACT samples, the concordance varied between 49% and 99.0%, while concordance for the DNA samples varied between 35.7% to 99%. These percentages of concordance were calculated in respect to the maximum possible score for each set of submitted results (which was the sum of total possible points for the number and type of analyses performed by that participant) (Table 13). The descriptions of analysis-specific results are provided in the following sections.

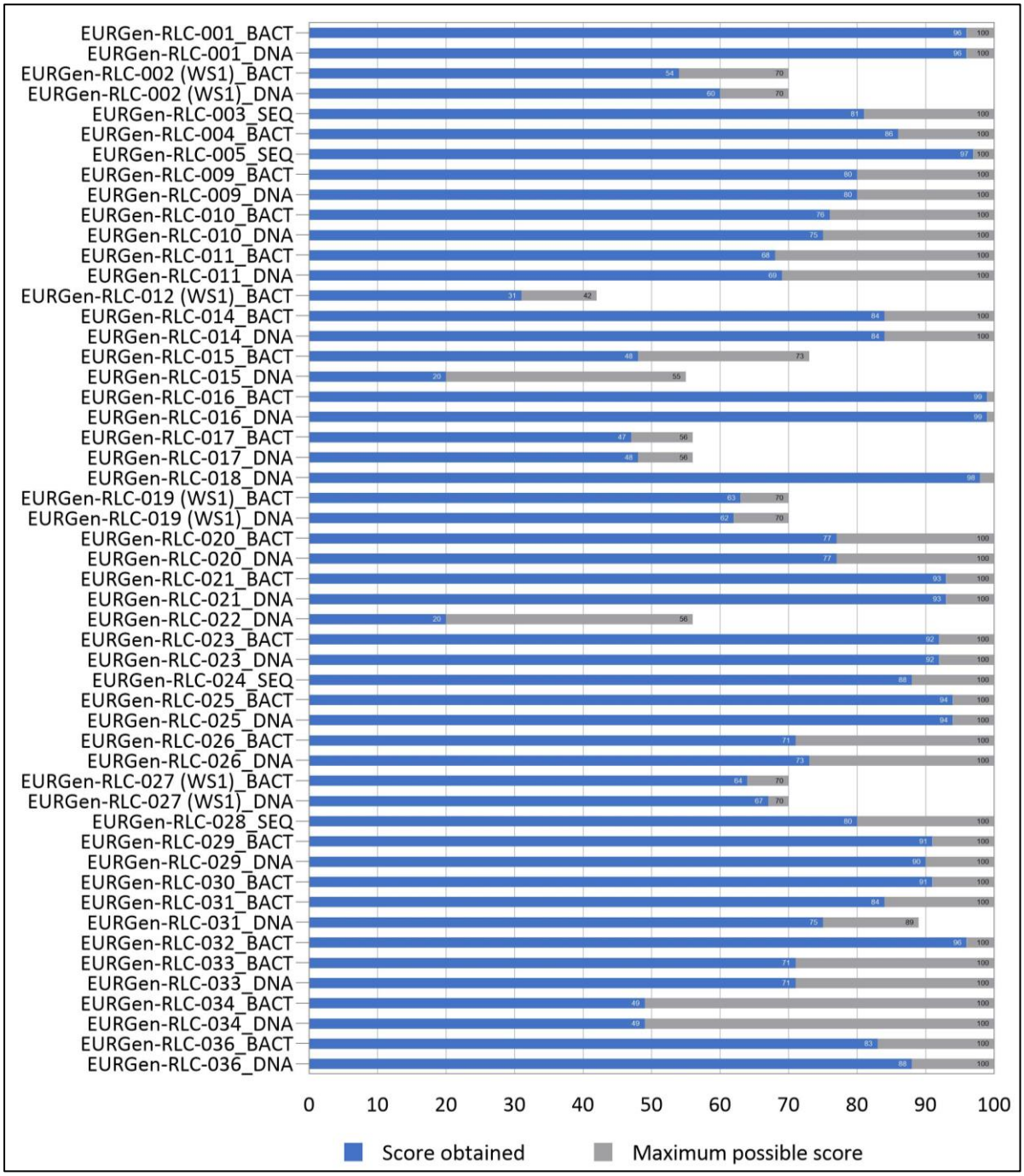


Figure 5. Concordance between submitted and expected results for all the analysis included in the EURGen-RefLabCap 2024 EQA, for each participant and for each type of dataset (BACT, DNA or SEQ). The maximum possible score for each participant varies depending on the number of analyses that they performed and for how many strains they performed those analyses. WS1: Participants only analysed material belonging to the WS1 (*E. coli and K. pneumoniae*).

Table 13. Maximum possible scores and scores obtained by each participant and for each type of dataset (BACT, DNA or SEQ), for each type of bioinformatics analysis included in the EURGen-RefLabCap 2024 EQA, and in total

Analysis		**Prediction of MLST**			**Detection of plasmid replicons**			**Detection of genetic AMR determinants**			**Prediction of AMR profiles**			**Totals**		
Participants	Sample type	Maximum possible score	ObtainedScore	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)
EURGen-RLC-001	BACT	4	4	100	11	10	90.9	41	40	97.6	44	42	95.5	100	96	96
	DNA	4	4	100	11	10	90.9	41	40	97.6	44	42	95.5	100	96	96
EURGen-RLC-002 (WS1)	BACT	2	2	100	11	7	63.6	29	19	65.5	28	26	92.9	70	54	77.1
	DNA	2	2	100	11	8	72.7	29	23	79.3	28	27	96.4	70	60	85.7
EURGen-RLC-003	SEQ	4	4	100	11	10	90.9	41	28	68.3	44	39	88.6	100	81	81
EURGen-RLC-004*	BACT	4	4	100	11	9	81.8	41	34	82.9	44	39	88.6	100	86	86
EURGen-RLC-005	SEQ	4	4	100	11	9	81.8	41	41	100	44	43	97.7	100	97	97
EURGen-RLC-009	BACT	4	4	100	11	10	90.9	41	30	73.2	44	36	81.8	100	80	80
	DNA	4	4	100	11	10	90.9	41	30	73.2	44	36	81.8	100	80	80
EURGen-RLC-010	BACT	4	4	100	11	11	100	41	33	80.5	44	28	63.6	100	76	76
	DNA	4	4	100	11	11	100	41	33	80.5	44	27	61.4	100	75	75
EURGen-RLC-011	BACT	4	4	100	11	7	63.6	41	24	58.5	44	33	75	100	68	68
	DNA	4	4	100	11	7	63.6	41	24	58.5	44	34	77.3	100	69	69
EURGen-RLC-012 (WS1)	BACT	2	2	100	11	7	63.6	29	22	75.9	NA	NA	NA	42	31	73.8
EURGen-RLC-014	BACT	4	4	100	11	10	90.9	41	34	82.9	44	36	81.8	100	84	84
	DNA	4	4	100	11	11	100	41	33	80.5	44	36	81.8	100	84	84
EURGen-RLC-015	BACT	4	3	75	11	7	63.6	41	24	58.5	17	14	82.4	73	48	65.8
	DNA	3	2	66.7	11	7	63.6	41	11	26.8	NA	NA	NA	55	20	36.4

Analysis		Prediction of MLST			Detection of plasmid replicons			Detection of genetic AMR determinants			Prediction of AMR profiles			Totals		
Participants	Sample type	Maximum possible score	ObtainedScore	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)
EURGen-RLC-016	BACT	4	3	75	11	11	100	41	41	100	44	44	100	100	99	99
	DNA	4	3	75	11	11	100	41	41	100	44	44	100	100	99	99
EURGen-RLC-017	BACT	4	4	100	11	6	54.5	41	37	90.2	NA	NA	NA	56	47	83.9
	DNA	4	4	100	11	6	54.5	41	38	92.7	NA	NA	NA	56	48	85.7
EURGen-RLC-018*	DNA	4	4	100	11	11	100	41	40	97.6	44	43	97.7	100	98	98
EURGen-RLC-019 (WS1)	BACT	2	2	100	11	11	100	29	23	79.3	28	27	96.4	70	63	90
	DNA	2	2	100	11	11	100	29	22	75.9	28	27	96.4	70	62	88.6
EURGen-RLC-020	BACT	4	4	100	11	5	45.5	41	34	82.9	44	34	77.3	100	77	77
	DNA	4	3	75	11	4	36.4	41	36	87.8	44	34	77.3	100	77	77
EURGen-RLC-021	BACT	4	4	100	11	11	100	41	35	85.4	44	43	97.7	100	93	93
	DNA	4	4	100	11	11	100	41	35	85.4	44	43	97.7	100	93	93
EURGen-RLC-022*	DNA	4	4	100	11	0	0	41	16	39	NA	NA	NA	56	20	35.7
EURGen-RLC-023	BACT	4	4	100	11	11	100	41	35	85.4	44	42	95.5	100	92	92
	DNA	4	4	100	11	11	100	41	35	85.4	44	42	95.5	100	92	92
EURGen-RLC-024	SEQ	4	4	100	11	9	81.8	41	37	90.2	44	38	86.4	100	88	88
EURGen-RLC-025	BACT	4	4	100	11	9	81.8	41	41	100	44	40	90.9	100	94	94
	DNA	4	4	100	11	9	81.8	41	41	100	44	40	90.9	100	94	94
EURGen-RLC-026	BACT	4	4	100	11	10	90.9	41	27	65.9	44	30	68.2	100	71	71
	DNA	4	4	100	11	11	100	41	27	65.9	44	31	70.5	100	73	73
EURGen-RLC-027 (WS1)	BACT	2	2	100	11	9	81.8	29	26	89.7	28	27	96.4	70	64	91.4

Analysis		Prediction of MLST			Detection of plasmid replicons			Detection of genetic AMR determinants			Prediction of AMR profiles			Totals		
Participants	Sample type	Maximum possible score	ObtainedScore	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)	Maximum possible score	Obtained Score	Score (%)
	DNA	2	2	100	11	10	90.9	29	27	93.1	28	28	100	70	67	95.7
EURGen-RLC-028	SEQ	4	4	100	11	11	100	41	29	70.7	44	36	81.8	100	80	80
EURGen-RLC-029	BACT	4	3	75	11	11	100	41	34	82.9	44	43	97.7	100	91	91
	DNA	4	3	75	11	11	100	41	33	80.5	44	43	97.7	100	90	90
EURGen-RLC-030*	BACT	4	4	100	11	9	81.8	41	36	87.8	44	42	95.5	100	91	91
EURGen-RLC-031	BACT	4	3	75	11	7	63.6	41	34	82.9	44	40	90.9	100	84	84
	DNA	4	3	75	11	7	63.6	41	34	82.9	33	31	93.9	89	75	84.3
EURGen-RLC-032*	BACT	4	4	100	11	11	100	41	39	95.1	44	42	95.5	100	96	96
EURGen-RLC-033	BACT	4	4	100	11	11	100	41	21	51.2	44	35	79.5	100	71	71
	DNA	4	4	100	11	11	100	41	21	51.2	44	35	79.5	100	71	71
EURGen-RLC-034	BACT	4	4	100	11	11	100	41	27	65.9	44	7	15.9	100	49	49
	DNA	4	4	100	11	11	100	41	27	65.9	44	7	15.9	100	49	49
EURGen-RLC-036	BACT	4	4	100	11	2	18.2	41	37	90.2	44	40	90.9	100	83	83
	DNA	4	4	100	11	4	36.4	41	40	97.6	44	40	90.9	100	88	88
Averages		**NA**	**3.5**	**95.5**	**NA**	**8.9**	**81.3**	**NA**	**31.3**	**79.6**	**NA**	**34.8**	**85.2**	**NA**	**75.3**	**81.7**

NA: Not applicable
WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*).
* The laboratory only submitted results for either BACT or DNA samples, and not both.

3.4. Prediction of multilocus sequence types

Participants used both publicly available and commercial software and/or databases for prediction of the MLST. The most reported software was CGE MLST and its respective database. A full description of the methods reported by the participants is provided in Appendix 3.

Overall, 31 laboratories submitted the results for the prediction of MLST and 21 laboratories submitted results for both BACT and DNA samples. Four laboratories submitted results for only BACT samples, while two laboratories submitted results for only DNA samples. Four laboratories submitted results for the sequence files provided by the EQA organizer.

In total, out of 208 possible MLST result submissions, 193 (92.8%) were submitted by all participating laboratories. In total, 27 of 31 participating laboratories submitted results for all four test strains. Four participants (EURGen-RLC-002, EURGen-RLC-012, EURGen-RLC-019 and EURGen-RLC-027) did not submit MLST results for WS2 pathogens, while one participant (EURGen-RLC-012) did not submit results for WS1 pathogens. In addition, one participant (EURGen-RLC-015) did not submit results for the test strain EURGen-2024-02 (*P. aeruginosa*).

The 193 submitted MLST predictions included predictions for strains EURGen-2024-01 (n=52), EURGen-2024-02 (n=44), EURGen-2024-03 (n=45) and EURGen-2024-04 (n=52) (Table 14, Figure 6). Of the submitted 193 MLST predictions, 184 were correct (95.5%).

All MLST predictions submitted for the strains EURGen-2024-01 and EURGen-2024-04 were correct. In total, nine incorrect MLST predictions (4.6%) were submitted, and all these incorrect results were submitted for strain EURGen-2024-03 (*A. baumannii*).

Overall, 26 participants correctly identified the ST of all strains for which they submitted results. Participants obtained between 66.7% to 100% of their maximum possible scores. The average concordance between expected and submitted results was 95.5% (Table 13, Figure 5).

Table 14. Distribution of submitted results regarding the prediction of MLST in the EURGen-RefLabCap 2024 EQA

Test strains	**Correct ST**	**Incorrect ST**	**Empty ST**	**Total**
EURGen-2024-01 (*E. coli*)	52	0	0	52
EURGen-2024-02 (*P. aeruginosa*)	44	0	8	52
EURGen-2024-03 (*A. baumannii)*	36	9	7	52
EURGen-2024-04 (*K. pneumoniae*)	52	0	0	52
Total	**184**	**9**	**15**	**208**

Figure 6. Distribution of submitted results regarding the prediction of ST in the EURGen-RefLabCap 2024 EQA, for each participant and for each type of dataset (BACT, DNA or SEQ). WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*).

The prediction of ST for EURGen-2024-01 (*E. coli*), EURGen-2024-04 (*K. pneumoniae*), and EURGen-2024-02 (*P. aeruginosa*) was in full concordance with the expected results. However, there were nine discordances reported for the strain EURGen-2024-03 (*A. baumannii*). All these discordances were due to the use of Pasteur MLST scheme for *A. baumannii* by the participants, as opposed to the Oxford scheme which was proposed for this EQA in the protocol.

In all these cases the Pasteur scheme for *A. baumannii* resulted in ST 764, which is correct for this strain if using that scheme. However, the participants were given a score of "0" since the STs submitted by the laboratories do not match the ST in the expected results.

For the self-evaluation, it should be considered that these discrepancies do not represent a flaw in the bioinformatics methods used by the participants but were due to not following the instructions described in the 2024 EQA protocol. The bioinformatics capacity and knowledge required for using either MLST scheme is the same, but it is important to understand that participants should adhere to analysis and reporting rules described in the EQA protocol, to strengthen the best-practice of ensuring that their analysis and reporting performed during routine work also follows the respective frameworks. The EQA providers do not recommend a specific MLST scheme for routine analysis outside the EQA activity and participants should adhere to their local guidelines.

3.5. Detection of plasmid replicons

Participants used both publicly available and commercial software and/or databases for detection of plasmid replicons. The most reported software was CGE PlasmidFinder and its respective database. A full description of the methods reported by the participants is provided in Appendix 4.

Overall, 31 laboratories submitted the results for the detection of plasmid replicons and 21 laboratories submitted results for both BACT and DNA samples. Four laboratories submitted results for only BACT samples, while two laboratories submitted results for only DNA samples. Four laboratories submitted results for the sequence files provided by the EQA organizer.

In total, 104 sets (31 laboratories) of results were submitted regarding the detection of plasmid replicons. The submitted results were distributed equally between the two strains EURGen-2024-01 (*E. coli*) and EURGen-2024-04 (*K. pneumoniae*) (n=52 sets of results per strain). For EURGen-2024-02 (*P. aeruginosa*) and EURGen-2024-03 (*A. baumannii*) no plasmid replicons were expected, and participants did not report any replicons for these strains.

Of the 104 sets of results submitted for the detection of plasmid replicons, 49% were fully correct (n=51). Additionally, in 34.6% of the sets of results (n=36) certain expected plasmid replicons were missing, and in 1% of the submitted results (n=1) unexpected replicons that were not part of the expected results were reported. In some of these cases, the sets of results were missing certain expected replicons and simultaneously contained unexpected replicons (15.4% or n=16) (Table 15, Figure 7).

Table 15. Distribution of submitted sets of results regarding the detection of plasmid replicons in the EURGen-RefLabCap 2024 EQA

Test strain	Correct replicons	Only missing replicons	Only unexpected replicons	Missing and un-expected replicons	Total
EURGen-2024-01 (*E. coli*)	26	17	1	8	**52**
EURGen-2024-02 (*P. aeruginosa*)*	NA	NA	NA	NA	**NA**
EURGen-2024-03 (*A. baumannii)**	NA	NA	NA	NA	**NA**
EURGen-2024-04 (*K. pneumoniae*)	25	19	0	8	**52**
Total	**51**	**36**	**1**	**16**	**104**

NA: Not applicable

* There were no expected plasmid replicons for the strain, therefore it was not possible for participants to report any "Correct replicon" or to miss any expected replicon, or to submit any unexpected replicon.

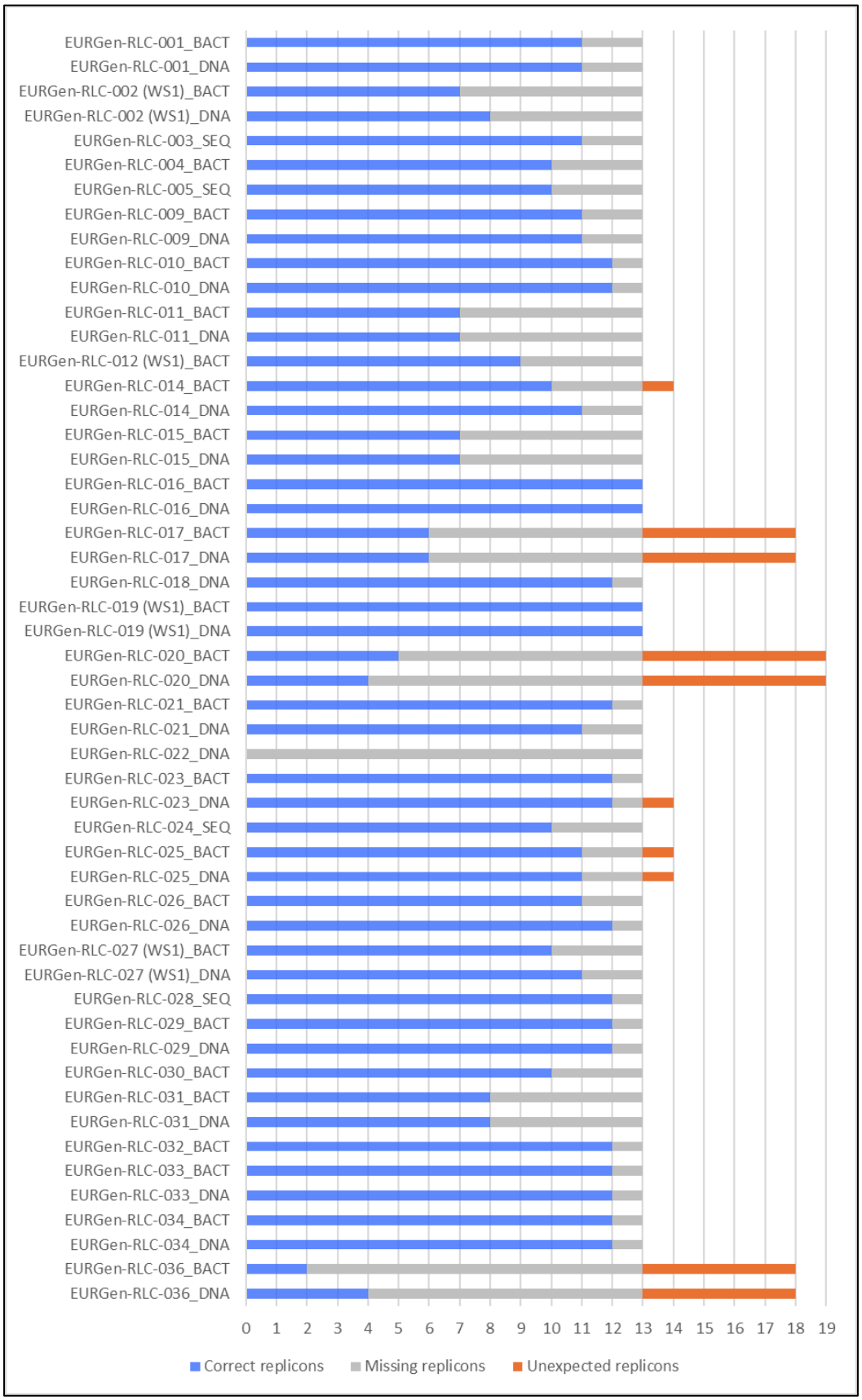


Figure 7. Distribution of submitted results regarding the detection of plasmid replicons in the EURGen-RefLabCap 2024 EQA, for each participant and for each type of dataset (BACT, DNA or SEQ). WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*).

Overall, the participants obtained between 0 and 11 points for the detection of plasmid replicons, which corresponded to 0% to 100 % of their maximum possible scores (Table 13, Figure 5). The average concordance between expected and submitted results was 81.3%. Two participants (four sets of results) correctly identified all expected replicons for both strains, without reporting any unexpected replicon.

For strain EURGen-2024-01 (*E. coli*), participants were expected to detect five plasmid replicons (ColpEC648, IncFIA, IncFIB(AP001918), IncFII, IncI1-I(Alpha)). The replicons ColpEC648 and IncFII were missing in 16 sets of results each, while IncI1-I(Alpha) was missing in 8 sets of results. There were eight more cases of other missing replicons from the submitted results. Overall, all the expected replicons were correctly reported in 26 sets of results (50%), while five results correctly reported all expected replicons except IncFII. The total number of missing replicons in all submitted sets of results was 48. Moreover, four unexpected replicons (IncFIC(FII) (n=8), IncI(Gamma) (n=6), IncFIB(K) (n=2) and Col(pHAD28) (n=1)) were submitted in nine sets of results. The total number of unexpected replicons throughout all sets of submitted results was 17. A complete description of the concordances and discordances between the expected plasmid replicons and the results submitted by participants is provided in Table 16.

Table 16. Results of the detection of plasmid replicons for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-01 (*E. coli*)

		Expected					Unexpected						
Laboratories	**Sample type**	ColpEC648	IncFIA	IncFIB(AP001918)	IncFII	IncI1-I(Alpha)	Col(pHAD28)	IncFIB(K)	IncFIC(FII)	IncI(Gamma)	**Correct (nr.)**	**Missing (nr.)**	**Unexpected (nr.)**
EURGen-RLC-001	BACT	x	x	x	-	x					4	1	0
	DNA	x	x	x	-	x					4	1	0
EURGen-RLC-002	BACT	x	-	x	-	x					3	2	0
	DNA	x	-	x	-	x					3	2	0
EURGen-RLC-003[b]	SEQ	-	x	x	x	x					4	1	0
EURGen-RLC-004[c]	BACT	-	x	x	x	x					4	1	0
EURGen-RLC-005[b]	SEQ	-	x	x	x	x					4	1	0
EURGen-RLC-009	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-010	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-011	BACT	x	x	x	-	x					4	1	0
	DNA	x	x	x	-	x					4	1	0
EURGen-RLC-012[c]	BACT	-	x	x	x	-					3	2	0
EURGen-RLC-014	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-015	BACT	-	x	x	-	x					3	2	0
	DNA	-	x	x	-	x					3	2	0
EURGen-RLC-016	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-017	BACT	-	x	x	x	-			x	x	3	2	2
	DNA	-	x	x	x	-			x	x	3	2	2
EURGen-RLC-018[b]	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-019	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-020	BACT	-	x	-	x	-		x	x	x	2	3	3
	DNA	-	x	-	-	-		x	x	x	1	4	3
EURGen-RLC-021	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-022[b]	DNA	-	-	-	-	-					0	5	0
EURGen-RLC-023	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x	x				5	0	1
EURGen-RLC-024[b]	SEQ	-	x	x	x	x					4	1	0
EURGen-RLC-025	BACT	x	x	x	-	x			x		4	1	1
	DNA	x	x	x	-	x			x		4	1	1
EURGen-RLC-026	BACT	x	x	x	-	x					4	1	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-027	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-028[b]	SEQ	x	x	x	x	x					5	0	0
EURGen-RLC-029	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-030[c]	BACT	x	x	x	x	x					5	0	0
EURGen-RLC-031	BACT	-	x	x	-	x					3	2	0
	DNA	-	x	x	-	x					3	2	0
EURGen-RLC-032[c]	BACT	x	x	x	x	x					5	0	0
EURGen-RLC-033	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-034	BACT	x	x	x	x	x					5	0	0
	DNA	x	x	x	x	x					5	0	0
EURGen-RLC-036	BACT	-	x	-	-	-			x	x	1	4	2
	DNA	-	x	-	x	-			x	x	2	3	2
Correct (nr.)		36	49	47	36	44	NA	NA	NA	NA		**Total**	
Missing or UN (nr.)		16	3	5	16	8	1	2	8	6	212	48	17

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): Plasmid replicon reported
Cells shaded in red (-): Plasmid replicon missing
Cells shaded in orange (x): Unexpected plasmid replicon reported
[b] The laboratories that analysed sequencing data provided by the EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both

For strain EURGen-2024-04 (*K. pneumoniae*), participants were expected to detect six plasmid replicons (Col440II, IncFIB(K), IncFII, IncFII(K), IncN4, IncX3), and two additional replicons (Col(pHAD28), ColKP3) were part of the expected results but not mandatory to report. Of 52 submissions by 31 participants, 25 sets of submissions (48%) correctly reported all expected replicons. The commonly missed expected replicons were IncFII(K) and IncN4 that were not reported in 22 (42%) and 16 (31%) submissions, respectively. Among the non-mandatory expected replicons, Col(pHAD28) was reported in 10 (19.2%) submissions, while ColKP3 was reported in 33 (63.5%) submissions. Furthermore, seven unexpected plasmid replicons (Col440I, ColRNAI, IncFIA, IncFIC(FII), IncFII(pAR0022), IncFII(pKP91) and IncN) were reported in seven submissions. The total number of unexpected replicons throughout all sets of submitted results was 29 (Table 17).

Table 17. Results of the detection of plasmid replicons for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-04 (*K. pneumoniae*)

		Expected								Unexpected										
Laboratories	**Sample type**	Col440II	IncFIB(K)	IncFII	IncFII(K)	IncN4	IncX3	ColKP3[a]	Col(pHAD28)[a]	Col440I	ColRNAI	IncFIA	IncFIC(FII)	IncFII(pAR0022)	IncFII(pKP91)	IncN	**Correct (nr.)**	**Missing (nr.)**	**Unexpected (nr.)**	**Expected non-mandatory (nr.)**
EURGen-RLC-001	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-002	BACT	x	-	x	-	x	x	-	-								4	2	0	0
	DNA	x	x	x	-	x	x	-	-								5	1	0	0
EURGen-RLC-003[b]	SEQ	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-004[c]	BACT	x	x	x	x	-	x	x	-								5	1	0	1
EURGen-RLC-005[b]	SEQ	-	x	x	x	x	x	x	-								5	1	0	1
EURGen-RLC-009	BACT	x	x	x	-	x	x	x	-								5	1	0	1
	DNA	x	x	x	-	x	x	x	-								5	1	0	1
EURGen-RLC-010	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-011	BACT	x	-	x	-	-	x	-	-								3	3	0	0
	DNA	x	-	x	-	-	x	-	-								3	3	0	0
EURGen-RLC-012[c]	BACT	x	x	x	-	-	x	x	x								4	2	0	2
EURGen-RLC-014	BACT	-	x	x	x	x	x	-	-	x							5	1	1	0
	DNA	x	x	x	x	x	x	-	-								6	0	0	0
EURGen-RLC-015	BACT	x	x	x	-	-	x	-	-								4	2	0	0
	DNA	x	x	x	-	-	x	-	-								4	2	0	0
EURGen-RLC-016	BACT	x	x	x	x	x	x	x	x								6	0	0	2
	DNA	x	x	x	x	x	x	x	x								6	0	0	2
EURGen-RLC-017	BACT	-	x	x	-	-	x	-	-		x	x	x			x	3	3	4	0
	DNA	-	x	x	-	-	x	-	-		x	x	x			x	3	3	4	0
EURGen-RLC-018[b]	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-019	BACT	x	x	x	x	x	x	x	x								6	0	0	2
	DNA	x	x	x	x	x	x	x	x								6	0	0	2
EURGen-RLC-020	BACT	-	x	x	-	-	x	-	-		x	x	x			x	3	3	4	0
	DNA	-	x	x	-	-	x	-	-		x	x	x			x	3	3	4	0
EURGen-RLC-021	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	-	-								6	0	0	0
EURGen-RLC-022[b]	DNA	-	-	-	-	-	-	-	-								0	6	0	0
EURGen-RLC-023	BACT	x	x	x	x	x	x	-	x								6	0	0	1
	DNA	x	x	x	x	x	x	-	x								6	0	0	1
EURGen-RLC-024[b]	SEQ	x	x	x	x	-	x	-	x								5	1	0	1
EURGen-RLC-025	BACT	x	x	x	-	x	x	x	x					x	x		5	1	2	2
	DNA	x	x	x	-	x	x	x	x					x	x		5	1	2	2
EURGen-RLC-026	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-027	BACT	x	-	x	-	x	x	x	-								4	2	0	1
	DNA	-	x	x	x	x	x	x	-								5	1	0	1
EURGen-RLC-028[b]	SEQ	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-029	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-030[c]	BACT	x	-	x	-	x	x	x	-								4	2	0	1
EURGen-RLC-031	BACT	x	x	x	-	-	x	x	-								4	2	0	1
	DNA	x	x	x	-	-	x	x	-								4	2	0	1
EURGen-RLC-032[c]	BACT	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-033	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-034	BACT	x	x	x	x	x	x	x	-								6	0	0	1
	DNA	x	x	x	x	x	x	x	-								6	0	0	1
EURGen-RLC-036	BACT	-	-	-	-	-	x	-	-		x	x	x			x	1	5	4	0
	DNA	-	-	x	-	-	x	-	-		x	x	x			x	2	4	4	0
Correct (nr.)		42	44	50	30	36	51	33	10	NA	NA	NA	NA	NA	NA	NA			**Total**	
Missing or UN (nr.)		10	8	2	22	16	1	19	42	1	6	6	6	2	2	6	253	59	29	43

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): Plasmid replicon reported
Cells shaded in red (-): Plasmid replicon missing
Cells shaded in orange (x): Unexpected plasmid replicon reported
[a] Expected but non-mandatory to report
[b] The laboratories that analysed sequencing data provided by the EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both

Overall, nearly half of the submitted sets of replicons were fully correct. However, 50% of submitted sets of results were missing certain expected replicons. This is most likely due to the participants' choice of thresholds, which potentially were stricter than those used to generate the expected results (which were minimum identity of 90% and minimum coverage of 90%). Of note, the choice of thresholds to generate expected results was subjective and the use of different thresholds is not necessarily incorrect. Additionally, the reason for missing replicons might be due to the use of different bioinformatics tools or databases than the ones used for generating expected results. For example, some of the participants that missed certain expected results were using Ridom SeqSphere+ and/or MOB-suite (Appendix 4). These programs use different algorithms and databases for the detection of replicons and may not be able to detect certain replicons or may also detect other replicons which are not part of the expected results.

Two laboratories reported the expected replicon IncFIB(K) only in the DNA samples and not in the BACT samples. This plasmid carried multiple AMR genes including *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, and *aac(6')-Ib-cr* (or closely related variants) which were also not reported by these laboratories in BACT samples. These observations suggest that this plasmid was lost during preparation, transport or handling of the live cultures. It is important to note that the loss of plasmid is a random and rare occurrence which cannot be controlled by the participants.

For the strain EURGen-2024-04 (*K. pneumoniae*) two replicons (Col(pHAD28) and ColKP3) were considered as expected but non-mandatory to report. This was due to the fact that these replicons could only be detected with one bioinformatics tool, or only detected in one type of sequence. The most commonly missed non-mandatory replicon was Col(pHAD28) which was missing in 42 out of 52 sets of submitted results. While preparing the expected results, Col(pHAD28) was not detected in the long-read sequencing data. Moreover, all laboratories that sequenced genomes with ONT had lower coverage on the plasmids due to low overall yield, which may result in poor coverage for some plasmids. Consequently, plasmids in such sequences may not be detected. The other expected non-mandatory replicon ColKP3 was only detected in FASTQ files and not detected in the FASTA files while preparing the expected results, thus it is not unforeseen that this replicon was also missed by most of the participants that used FASTA files for the plasmid detection. This difference in expected results furthermore strengthens the observation that the assembly process might fail to properly capture sequences that were present in raw data, for example due to a different depth of coverage than the one of the genomic DNA, or due to sequencing of more fragmented plasmid DNA (because of the DNA extraction process which often is not optimized for adequate plasmid extraction). Thus, it is important to consider the goals of each analysis before selecting a particular technology or bioinformatics approach.

The participants reported unexpected replicon in 16.3% sets of results (n=14), less frequently than missing expected replicons. Some of these discordances might be because different tools or databases report different replicons for the same sequence. For example, PlasmidFinder reports IncN4 while the MOB-suite reports IncN for the same plasmid sequence (100% sequence similarity), and laboratories that reported IncN used MOB-suite for plasmid replicon detection. Some of these discordances might be due to reporting of several similar replicons that appeared in the output. This might be due to the lack of knowledge of differences between similar replicon types or insufficient scrutiny of the results from the bioinformatics tools. A more careful analysis of the results is needed in such a way that, when receiving several options for the same genomic sequence, only the replicon with highest percentage of identity must be reported and the other replicons for the same location must then be discarded.

3.6. Detection of genes and chromosomal point mutations mediating AMR

Participants used both publicly available and commercial bioinformatics tools and/or databases for detection of the genetic determinants mediating AMR. The most reported software was CGE ResFinder and its respective database. A full description of the methods reported by the participants is provided in Appendix 5.

Overall, 31 laboratories submitted the results for the detection of AMR determinants and 21 laboratories submitted results for both BACT and DNA samples. Six laboratories only analysed either BACT or DNA samples, while four laboratories analysed the sequence files provided by the EQA organizer.

In total, 193 sets of results were submitted regarding the detection of genetic determinants mediating AMR, by all participating laboratories. For EURGen-2024-01 (*E. coli*) and EURGen-2024-04 (*K. pneumoniae*), all 31 participants submitted the results for the detection of AMR genes and PMs, while 27 participants submitted the results for EURGen-2024-02 (*P. aeruginosa*) and EURGen-2024-03 (*A. baumannii*) (Table 18, Figure 8).

Of the 193 sets of results submitted for detection of genetic determinants mediating AMR, 9.8% were fully correct (n=19). Additionally, certain expected genetic determinants were missing in 15.5% of the sets of results (n=30), and in 21.8% of the submitted results (n=42) unexpected genetic determinants that were not part of the expected results were reported. Moreover, in 52.8% (n=102) of sets of results, certain expected determinants were missing and simultaneously contained unexpected genetic determinants of AMR (Table 18, Figure 8).

Table 18. Distribution of submitted results regarding the detection of genetic determinants of AMR in the EURGen-RefLabCap 2024 EQA

Test strain	Correct determinants	Only missing determinants	Only unexpected determinants	Missing and un-expected determinants	Total
EURGen-2024-01 (*E. coli*)	3	9	9	31	**52**
EURGen-2024-02 (*P. aeruginosa*)	4	7	7	26	**44**
EURGen-2024-03 (*A. baumannii)*	10	8	15	12	**45**
EURGen-2024-04 (*K. pneumoniae*)	2	6	11	33	**52**
Total	**19**	**30**	**42**	**102**	**193**

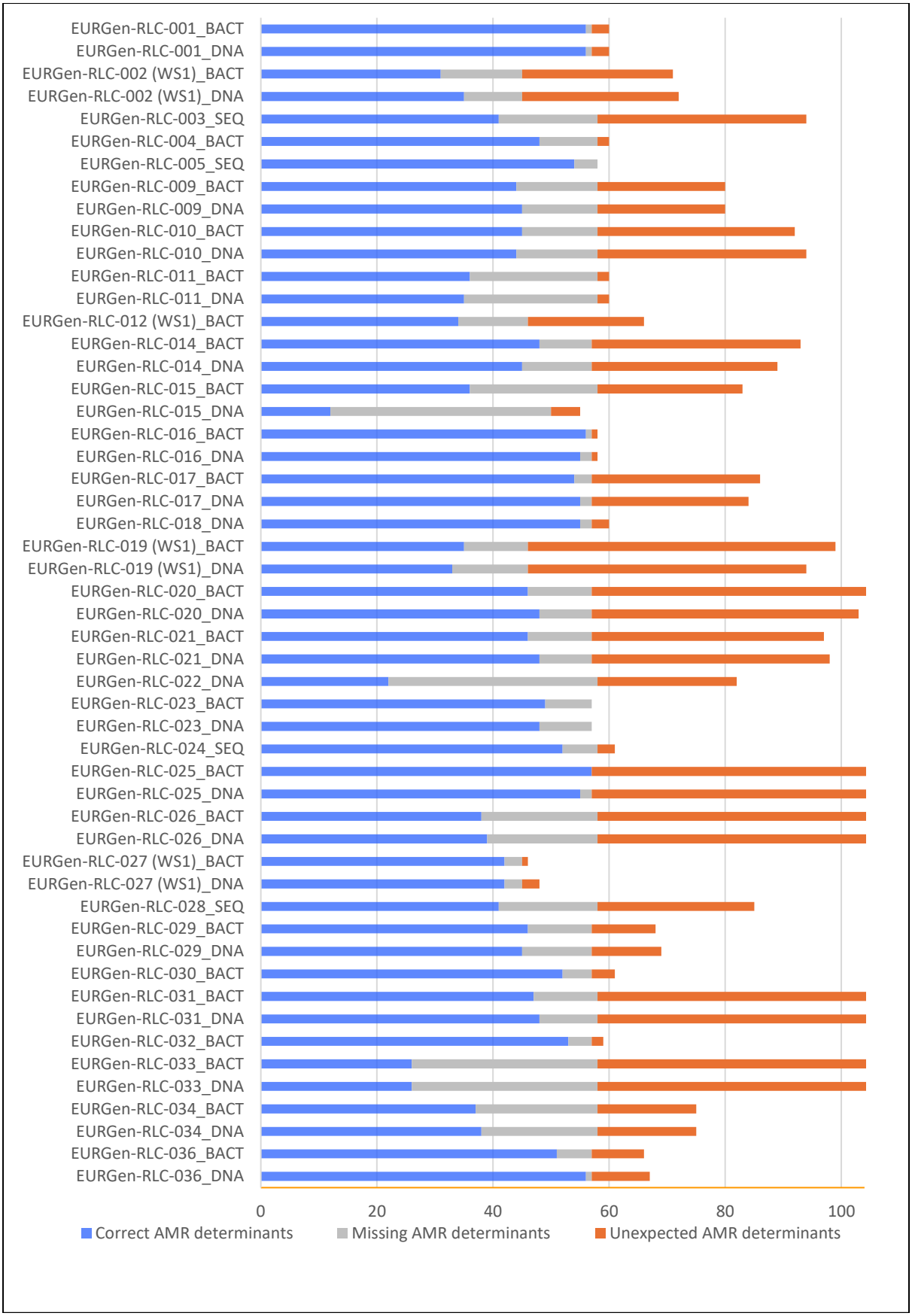


Figure 8. Distribution of submitted results regarding the detection of genetic determinants mediating AMR in the EURGen-RefLabCap 2024 EQA, for each participant and for each type of dataset (BACT, DNA or SEQ). WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*).

Overall, the participants obtained between 15 and 41 points for the detection of genetic determinants of AMR. The participants obtained between 36.6% to 100% scores of their maximum possible scores (Table 13, Figure 5). The average concordance between expected and submitted results was 79.4%. None of the participants reported all the correct expected genetic determinants of AMR for all the analysed strains. Similarly, all participants identified at least one of the expected determinants.

For strain EURGen-2024-01 (*E. coli*), participants were expected to detect 10 genes (*aac(3)-IIa* or *aac(3)-IIe,* $bla_{CTX\text{-}M\text{-}65}$, $bla_{TEM\text{-}1}$ or $bla_{TEM\text{-}1A}$ or $bla_{TEM\text{-}1B}$ or $bla_{TEM\text{-}1C}$ or $bla_{TEM\text{-}1D}$, *dfrA12, dfrA17, sul1, sul2, sul3, fosA3*, *mcr-1.1* or *mcr-1.26*) and six chromosomal PMs mediating AMR (*glpT* E448K, *gyrA* D87Y, *gyrA* S83L, *parC* S80I, *parE* S458A, *uhpT* E350Q). In total, 31 laboratories submitted 52 sets of results for the detection of genetic AMR determinants for this strain. Of these, 25 sets of results were submitted for BACT while 23 were submitted for DNA samples. The most reported expected AMR determinants in all sets of results were *fosA3* (n=50) and *mcr1.1* or *mcr-1.26* (n=50), followed by $bla_{CTX\text{-}M\text{-}65}$ (n=49) and $bla_{TEM\text{-}1}$ or its variants (n=49).The most frequently missed AMR determinant was the PM *uhpT* E350Q (n=27), followed by *glpT* E448K (n=26), *gyrA* D87Y (n=17), and *gyrA* S83L (n=16). There were 82 more cases where an expected AMR determinant was missing in the submitted sets of results. The total number of missing genetic determinants of AMR throughout all sets of submitted results was 168 (Table 19). Of all 52 sets of submitted results for EURGen-2024-01, 40 sets contained at least one unexpected AMR determinant. A total of 225 unexpected AMR determinants were reported in all submitted sets of results. Of these, the most reported unexpected AMR determinant was *aadA2* (n=25), followed by *aadA5* (n=24), *aadA1* (n=23), *aph(3')-Ia* (n=23) and *tet(A)* (n=20). Only three sets of results contained all expected AMR determinants without any unexpected genes or chromosomal PMs. A complete description of the concordances and discordances between the expected genetic determinants of AMR and the results submitted by participants is provided in Table 19.

Table 19. Results of the detection of genetic AMR determinants for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-01 (*E. coli*)

		Expected																Unexpected																																								
Laboratories	**Sample type**	*aac(3)-IIa**	*bla* CTX-M-65	*bla* TEM-1*	*dfrA12*	*dfrA17*	*fosA3*	*mcr-1.1**	*sul1*	*sul2*	*sul3*	*glpT* E448K	*uhpT* E350Q	*gyrA* D87Y	*gyrA* S83L	*parC* S80I	*parE* S458A	*aac(3)-IIc*	*aadA1*	*aadA2*	*aadA5*	*ampC* internalstop	*ant(3'')-Ia*	*ant(3'')-IIa*	*aph(3')-Ia*	*aph(3'')-Ia*	*aph(3')-Ib*	*aph(3'')-Ib*	*aph(3')-Id*	*aph(6)-Id*	*bla* CTX-M-1	*bla* CTX-M-63	*bla* EC	*bla* EC-8	*mcr1.11*	*qnrVC1*	*tet(A)*	*tet(M)*	*cyaA* S352T	*glpT* glpT_E448K	*glpT* T448K	*gyrA* D87Y,S83L	*gyrA* D97Y	*gyrA* gyrA_D87Y	*gyrA* gyrA_S83L	*gyrA* p.D87Y	*gyrA* p.S83L	*gyrA* S83L;D87Y	*parC* p.S80I	*parC* parC_S80I	*parE* p.S458A	*parE* parE_S458A	*uhpA* E350Q	*uhpT* uhpT_E350Q	**Correct (nr.)**	**Missing (nr.)**	**Unexpected (nr.)**
EURGen-RLC-001	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																						x																16	0	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																						x																16	0	1
EURGen-RLC-002	BACT	x	x	x	x	x	x	x	x	x	x	-	-	-	-	x	x			x	x		x		x			x									x	x										x							12	4	8
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	-	-	x	x		x	x	x		x		x			x									x	x										x							12	4	9
EURGen-RLC-003[b]	SEQ	x	x	x	-	-	x	x	x	x	x	-	-	x	x	x	x			x	x				x			x		x																									12	4	5
EURGen-RLC-004[c]	BACT	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x															x																							15	1	1
EURGen-RLC-005[b]	SEQ	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x																																						15	1	0
EURGen-RLC-009	BACT	x	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x																																						14	2	0
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x																																						14	2	0
EURGen-RLC-010	BACT	-	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x								x		x										x	x																	13	3	4
	DNA	-	-	x	x	x	x	x	x	x	x	-	-	x	x	x	x								x		x				x						x	x																	12	4	5
EURGen-RLC-011	BACT	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-																																						10	6	0
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-																																						10	6	0
EURGen-RLC-012[c]	BACT	x	x	x	x	x	-	x	x	x	x	-	-	x	x	x	-		x	x	x				x			x									x	x																	12	4	7
EURGen-RLC-014	BACT	-	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x				x			x															x												14	2	6
	DNA	-	x	-	-	x	x	x	x	x	x	x	x	-	x	x	x		x	x	x				x			x																											12	4	5
EURGen-RLC-015	BACT	-	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x																				x	x																	13	3	2
	DNA	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-																																						1	15	0
EURGen-RLC-016	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																																						16	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																																						16	0	0
EURGen-RLC-017	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x				x			x											x																16	0	6
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x				x			x											x																16	0	6
EURGen-RLC-018[c]	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x											x																											16	0	1
EURGen-RLC-019	BACT	x	x	x	x	x	x	x	x	x	x	x	x	-	-	-	-	x	x	x	x					x	x		x								x	x	x							x	x		x		x				12	4	14
	DNA	-	x	x	x	x	x	x	x	x	x	x	x	-	-	-	-		x	x	x				x		x											x	x							x	x		x		x				11	5	11
EURGen-RLC-020	BACT	x	x	x	x	x	x	x	-	-	-	-	x	x	x	x	x		x	x	x				x			x									x	x			x														12	4	8
	DNA	x	x	x	x	x	x	x	-	-	-	-	x	x	x	x	x		x	x	x				x			x									x	x			x														12	4	8
EURGen-RLC-021	BACT	-	x	x	x	x	x	x	-	-	-	x	x	x	x	x	x		x	x	x					x		x					x				x																		12	4	7
	DNA	x	x	x	x	x	x	x	-	-	-	x	x	x	x	x	x		x	x	x				x			x					x				x																		13	3	7
EURGen-RLC-022[c]	DNA	-	x	x	-	x	x	-	x	-	-	-	-	-	-	-	-							x						x					x	x	x																		5	11	5
EURGen-RLC-023	BACT	x	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x																																						14	2	0
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x																																						14	2	0
EURGen-RLC-024[b]	SEQ	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																						x																15	1	1
EURGen-RLC-025	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x			x			x						x			x	x	x																16	0	10
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x			x			x						x			x	x	x																16	0	10
EURGen-RLC-026	BACT	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-		x	x	x				x			x									x									x	x				x				10	6	9
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-		x	x	x				x			x									x									x	x				x				10	6	9
EURGen-RLC-027	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																																						16	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x																																				x		15	1	1
EURGen-RLC-028[b]	SEQ	x	x	x	x	x	x	x	-	x	x	-	-	x	x	x	x		x	x					x																														13	3	3
EURGen-RLC-029	BACT	x	x	x	x	-	x	x	x	x	x	x	x	-	-	x	x																						x			x													13	3	2
	DNA	x	x	x	x	-	x	x	x	x	x	x	x	-	-	x	x																						x			x													13	3	2
EURGen-RLC-030[c]	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																						x																16	0	1
EURGen-RLC-031	BACT	x	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x		x	x	x				x			x									x	x																	14	2	7
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	x	x	x	x		x	x	x				x			x									x	x																	14	2	7
EURGen-RLC-032[c]	BACT	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x																																						15	1	0
EURGen-RLC-033	BACT	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-		x	x	x				x			x					x				x	x		x				x	x					x		x		x	5	11	14
	DNA	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-		x	x	x				x			x					x				x	x		x				x	x					x		x		x	5	11	14
EURGen-RLC-034	BACT	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-		x	x	x																																		10	6	3
	DNA	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-		x	x	x																																		10	6	3
EURGen-RLC-036	BACT	x	x	-	x	x	x	x	x	x	-	x	x	x	x	x	x						x																																14	2	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x						x																																16	0	1
Correct (nr.)		43	49	49	45	46	50	50	44	44	43	26	25	35	36	40	39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		**Total**	
Missing or UN (nr.)		9	3	3	7	6	2	2	8	8	9	26	27	17	16	12	13	1	23	25	24	2	4	1	23	2	4	21	1	2	1	1	4	2	1	1	20	16	0	2	2	2	1	2	2	4	4	2	2	2	4	2	1	2	664	168	225

NA: Not applicable; UN: Unexpected

Cells shaded in green (x): Genetic AMR determinant reported

Cells shaded in red (-): Genetic AMR determinant missing

Cells shaded in orange (x): Unexpected genetic AMR determinant reported

[b] The laboratories that analysed only the sequencing data provided by EQA organizer

[c] The laboratories only analysed either BACT or DNA samples, and not both

* Either *aac(3)-IIa or aac(3)-IIe,* either *bla*$_{\text{TEM-1}}$ or *bla*$_{\text{TEM-1A}}$ or *bla*$_{\text{TEM-1B}}$ or *bla*$_{\text{TEM-1C}}$ or *bla*$_{\text{TEM-1D}}$, [c] either *mcr1.1* or *mcr-1.26*

For strain EURGen-2024-02 (*P. aeruginosa*), participants were expected to detect seven genes (*aph(3')-VI* or *aph(3')-Via, aac(6')-Ib* or *aac(6')-Ib-Hangzhou* or *aac(6')-Ib3* or *aac(6')-Ib4* or *aac(6')-Ib9, bla*$_{IMP-62}$*, bla*$_{NDM-1}$*, bla*$_{PME-1}$*, crpP*, *qnrVC1*) and one chromosomal PM mediating AMR (*gyrA* T83I). Additionally, four AMR genes (*ant(3'')-Ii-aac(6')-Iid* or *ant(3'')-Ih/aac(6')-Iid, aac(6')-Ib-cr* or *aac(6')-Ib-cr5, bla*$_{KBL-1}$, *qepA* or *qepA1* or *qepA2* or *qepA4*) and three PMs (*nalC* G71E*, nalC* S209R, *parC* S87L) were also expected but were non-mandatory to report. In total, 44 sets of results were submitted by 27 laboratories for the detection of AMR determinants for this strain. Of these, 21 sets of results were submitted for BACT while 19 were submitted for DNA samples. The expected gene *bla*$_{NDM-1}$ was reported by all laboratories in all the submitted sets of results. Other most reported genes in all sets of results were *qnrVC1* (n=41) and *bla*$_{IMP-62}$ (n=40). The expected gene *aac(6')-Ib* or its accepted variants was not reported in 15 sets of results, while *crpP* was missing in 18 sets of results. The expected PM *gyrA* T83I was not reported in 16 sets of results. There were 31 more cases where expected AMR determinants were not reported. The total number of missing genetic determinants of AMR throughout all sets of submitted results was 80 (Table 20). Four laboratories reported fully correct set of results with all expected AMR determinants and without any unexpected determinants. All sets of results contained at least one expected determinant of AMR. Among the non-mandatory expected AMR determinants, the most reported determinant was *parC* S87L (n=25), followed by *bla*$_{KBL-1}$ (n=6). The unexpected genetic determinants of AMR were reported in the majority of the submitted sets of results (n=33). The most reported unexpected AMR gene was *fosA* (n=25), followed by *bla*$_{CARB-2}$ (n=23), *bla*$_{OXA-486}$ (n=18), *aph(3')-IIb* (n=18), *bla*$_{PDC-5}$ (n=17) and *tet(G)* (n=15). The total number of unexpected genetic determinants of AMR throughout all sets of submitted results was 249 (Table 20).

Table 20. Results of the detection of genetic AMR determinants for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-02 (*P. aeruginosa*)

		Expected																		
Laboratories	**Sample type**	*aph(3')-VI**	*bla*$_{\text{IMP-62}}$	*bla*$_{\text{NDM-1}}$	*bla*$_{\text{PME-1}}$	*crpP*	*qnrVC1*	*aac(6')-Ib**	*gyrA* T83I	aac(6')-Ib-cr*[a]	*ant(3'')-Ii-aac(6')-Iid**[a]	*bla*$_{\text{KBL-1}}$[a]	*qepA**[a]	*nalC* G71E[a]	*nalC* S209R[a]	*parC* S87L[a]	**Correct (nr.)**	**Missing (nr.)**	**Unexpected (nr.)**	**Expected non-mandatory (nr.**
EURGen-RLC-001	BACT	x	x	x	x	-	x	x	x	-	-	-	-	-	-	x	7	1	0	1
	DNA	x	x	x	x	-	x	x	x	-	-	-	-	-	-	x	7	1	0	1
EURGen-RLC-003[b]	SEQ	x	x	x	x	x	x	x	-	-	-	-	-	-	-	-	7	1	4	0
EURGen-RLC-004[c]	BACT	-	x	x	x	x	-	-	x	-	-	-	-	-	-	x	5	3	0	1
EURGen-RLC-005[b]	SEQ	x	x	x	x	x	x	x	x	-	-	-	-	-	-	-	8	0	0	0
EURGen-RLC-009	BACT	-	x	x	-	x	x	-	-	-	-	-	-	-	-	-	4	4	2	0
	DNA	-	x	x	-	x	x	-	-	-	-	-	-	-	-	-	4	4	2	0
EURGen-RLC-010	BACT	x	x	x	x	x	x	x	-	-	-	x	-	-	-	-	7	1	12	0
	DNA	x	x	x	x	x	x	x	x	-	-	x	-	-	-	x	8	0	12	1
EURGen-RLC-011	BACT	-	x	x	x	-	x	-	-	-	-	-	-	-	-	-	4	4	1	0
	DNA	-	x	x	x	-	x	-	-	-	-	-	-	-	-	-	4	4	1	0
EURGen-RLC-014	BACT	-	x	x	-	-	-	-	x	-	-	-	-	-	-	x	3	5	9	1
	DNA	-	x	x	-	-	x	-	x	-	-	-	-	-	-	x	4	4	8	1
EURGen-RLC-015[c]	BACT	x	x	x	x	-	x	x	x	-	-	-	-	-	-	x	7	1	12	1
EURGen-RLC-016	BACT	x	x	x	x	x	x	x	x	-	x	-	-	-	-	x	8	0	0	1
	DNA	x	x	x	x	x	x	x	x	-	x	-	-	-	-	x	8	0	0	1
EURGen-RLC-017	BACT	x	x	x	-	-	x	-	-	-	-	-	-	-	-	-	4	4	5	0
	DNA	x	x	x	-	-	x	-	x	-	-	-	-	-	-	x	5	3	3	1
EURGen-RLC-018[c]	DNA	x	x	x	x	x	x	x	x	-	-	-	-	-	-	x	8	0	1	1
EURGen-RLC-020	BACT	-	x	x	x	x	x	-	x	-	-	x	-	-	-	x	6	2	16	1
	DNA	x	x	x	x	x	x	x	x	-	-	x	-	-	-	x	8	0	14	1
EURGen-RLC-021	BACT	x	x	x	x	x	x	x	x	-	-	-	-	-	-	x	8	0	10	1
	DNA	x	-	x	x	x	x	x	x	-	-	-	-	-	-	x	7	1	11	1
EURGen-RLC-022[c]	DNA	-	-	x	x	-	x	-	-	-	-	-	-	-	-	-	3	5	5	0
EURGen-RLC-023	BACT	x	x	x	-	-	x	x	x	-	-	-	-	-	-	x	6	2	0	1
	DNA	x	x	x	-	-	x	x	x	-	-	-	-	-	-	x	6	2	0	1
EURGen-RLC-024[b]	SEQ	-	x	x	x	x	x	-	x	-	-	-	-	-	-	-	6	2	0	0
EURGen-RLC-025	BACT	x	x	x	x	x	x	x	x	-	-	x	-	-	-	x	8	0	14	1
	DNA	x	x	x	x	x	x	x	x	-	-	x	-	-	-	x	8	0	13	1
EURGen-RLC-026	BACT	x	x	x	x	x	x	-	-	-	-	-	-	-	-	-	6	2	17	0
	DNA	x	x	x	x	x	x	-	-	-	-	-	-	-	-	-	6	2	17	0
EURGen-RLC-028[b]	SEQ	-	x	x	x	-	-	-	x	x	-	-	-	x	x	-	4	4	2	0
EURGen-RLC-029	BACT	x	x	x	-	x	x	x	x	-	-	-	-	-	-	x	7	1	2	1
	DNA	x	x	x	-	x	x	x	x	-	-	-	-	-	-	x	7	1	2	1
EURGen-RLC-030[c]	BACT	x	x	x	-	-	x	x	x	-	-	-	-	-	-	x	6	2	1	1
EURGen-RLC-031	BACT	x	x	x	x	-	x	x	-	x	-	-	-	-	-	-	6	2	6	0
	DNA	x	x	x	x	-	x	x	-	x	-	-	-	-	-	-	6	2	7	0
EURGen-RLC-032[c]	BACT	x	x	x	x	x	x	x	x	-	-	-	-	-	-	x	8	0	0	1
EURGen-RLC-033	BACT	x	x	x	x	-	x	x	-	-	-	-	-	-	-	-	6	2	14	0
	DNA	x	x	x	x	-	x	x	-	-	-	-	-	-	-	-	6	2	14	0
EURGen-RLC-034	BACT	-	-	x	x	x	x	x	-	-	-	-	-	-	-	-	5	3	4	0
	DNA	-	-	x	x	x	x	x	-	-	-	-	-	-	-	-	5	3	4	0
EURGen-RLC-036	BACT	x	x	x	x	x	x	x	x	-	-	-	-	-	-	x	8	0	2	1
	DNA	x	x	x	x	x	x	x	x	-	-	-	-	-	-	x	8	0	2	1
EURGen-RLC-002	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-012	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-019	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-027	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
Correct (nr.)		31	40	44	33	26	41	29	28	3	2	6	0	1	1	25	**Total**			
Missing or UN (nr.)		13	4	0	11	18	3	15	16	41	42	38	44	43	43	19	272	80	249	25

		Unexpected																																				
Laboratories	**Sample type**	*aadA1*	*aadA10*	*aadA11*	*aadA12*	*aadA15*	*aadA2*	*aadA2b*	*ant(3'')-Ib*	*ant(3'')-Ij/aac(6')-Ib*	*aph(3'')-Ib*	*aph(3')-IIb*	*aph(3')-VIb*	*aph(6)-Id*	*bla2*	*bla*$_{\text{ADC-262}}$	*blaCARB-2*	*bla*$_{\text{IMP-22}}$	*bla*$_{\text{OXA-396}}$	*bla*$_{\text{OXA-486}}$	*bla*$_{\text{OXA-494}}$	*bla*$_{\text{OXA-50}}$	*bla*$_{\text{PAO}}$	*bla*$_{\text{PDC-5}}$	*fosA*	*qnrVC10*	*sul1*	*tet*	*tet(G)*	*tmexC3*	tmexD	*tmexD3*	*TOprJ1*	*TOprJ3*	*gyrA gyrA_T83I*	*parC parC_S87L*	*gyrA S87L*	*parC* T83I
EURGen-RLC-001	BACT																																					
	DNA																																					
EURGen-RLC-003[b]	SEQ																x			x			x		x													
EURGen-RLC-004[c]	BACT																																					
EURGen-RLC-005[b]	SEQ																																					
EURGen-RLC-009	BACT																						x		x													
	DNA																						x		x													
EURGen-RLC-010	BACT			x		x	x				x						x			x				x	x		x			x		x	x					
	DNA			x		x	x				x				x		x			x				x	x		x			x			x					
EURGen-RLC-011	BACT											x																										
	DNA											x																										
EURGen-RLC-014	BACT																x			x				x	x	x			x	x		x	x					
	DNA																x			x				x	x				x	x		x	x					
EURGen-RLC-015[c]	BACT			x		x		x			x	x		x			x			x			x		x		x		x									
EURGen-RLC-016	BACT																																					
	DNA																																					
EURGen-RLC-017	BACT											x					x								x												x	x
	DNA											x					x								x													
EURGen-RLC-018[c]	DNA																							x														
EURGen-RLC-020	BACT	x		x			x			x	x	x	x	x			x			x				x	x				x	x	x		x					
	DNA	x		x			x				x	x		x			x			x				x	x				x	x		x	x					
EURGen-RLC-021	BACT						x					x					x			x				x	x				x	x		x		x				
	DNA						x					x					x	x		x				x	x				x	x		x	x					
EURGen-RLC-022[c]	DNA											x											x		x		x	x										
EURGen-RLC-023	BACT																																					
	DNA																																					
EURGen-RLC-024[b]	SEQ																																					
EURGen-RLC-025	BACT			x		x	x				x	x					x			x				x	x		x		x	x		x	x					
	DNA			x		x	x				x	x					x			x				x	x		x		x			x	x					
EURGen-RLC-026	BACT			x	x		x		x			x		x			x			x	x	x	x		x		x		x	x		x		x				
	DNA			x	x		x				x	x		x			x		x	x		x	x		x		x		x	x		x		x				
EURGen-RLC-028[b]	SEQ																						x									x						
EURGen-RLC-029	BACT																x							x														
	DNA															x	x																					
EURGen-RLC-030[c]	BACT																							x														
EURGen-RLC-031	BACT			x								x								x			x		x				x									
	DNA			x								x					x			x			x		x				x									
EURGen-RLC-032[c]	BACT																																					
EURGen-RLC-033	BACT			x		x	x					x					x			x				x	x				x	x		x	x		x	x		
	DNA			x		x	x					x					x			x				x	x				x	x		x	x		x	x		
EURGen-RLC-034	BACT		x			x		x									x																					
	DNA	x	x					x									x																					
EURGen-RLC-036	BACT																							x	x													
	DNA																							x	x													
EURGen-RLC-002	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
EURGen-RLC-012	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
EURGen-RLC-019	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
EURGen-RLC-027	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Correct (nr.)		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Missing or UN (nr.)		3	2	13	2	8	12	3	1	1	8	18	1	5	1	1	23	1	1	18	1	2	10	17	25	1	8	1	15	13	1	13	11	3	2	2	1	1

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): Genetic AMR determinant reported
Cells shaded in red (-): Genetic AMR determinant missing
Cells shaded in orange (x): Unexpected genetic AMR determinant reported
Cells shaded in grey (/): participant did not submit AMR genes and mutations

[a] Expected results but non mandatory to report
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both
[d] The laboratory did not submit results for EURGen-2024-02
[*] Either *aph(3')-VI* or *aph(3')-Via,* either *aac(6')-Ib* or *aac(6')-Ib-Hangzhou* or *aac(6')-Ib3* or *aac(6')-Ib4* or *aac(6')-Ib9*, either *ant(3'')-Ii-aac(6')-Iid* or *ant(3'')-Ih/aac(6')-Iid,* either *qepA* or *qepA1* or *qepA2* or *qepA4*

For strain EURGen-2024-03 (*A. baumannii*), participants were expected to detect two genes (*ant(2'')-Ia, bla*$_{OXA-23}$) and two chromosomal PMs (*gyrA* S81L*, parC* S84L) mediating AMR. Two chromosomal PMs were expected but non-mandatory to report (*parC* D105E*, parC* V104I). In total, 45 sets of results were submitted by 27 laboratories for the detection of AMR determinants for this strain. Of these, 21 sets of results were submitted for BACT while 20 were submitted for DNA samples. Ten sets of submitted results contained all the expected AMR determinants without any unexpected determinant. One set of results was missing all the expected determinants of AMR. The most reported expected AMR determinant was *ant(2'')-Ia* (n=44), followed by *bla*$_{OXA-23}$ (n=41). The expected PMs *gyrA S*81L and *parC* S84L were missing in 19 sets of results each. The total number of missing genetic determinants of AMR throughout all sets of submitted results was 43. Among the non-mandatory expected AMR determinants, almost all sets of results were missing *parC* D105E (n=42) and *parC* V104I (n=42). There were 217 unexpected AMR determinants reported in 27 sets of results. The most reported unexpected AMR gene was *bla*$_{CARB-2}$ (n=25), followed by *bla*$_{OXA-429}$ (n=23), *aph(3'')-Ib* (n=19), *aadA2* (n=19), *tet(G)* (n=18), and *tet(B)* (n=18). The total number of unexpected genetic determinants of AMR throughout all sets of submitted results was 217 (Table 21).

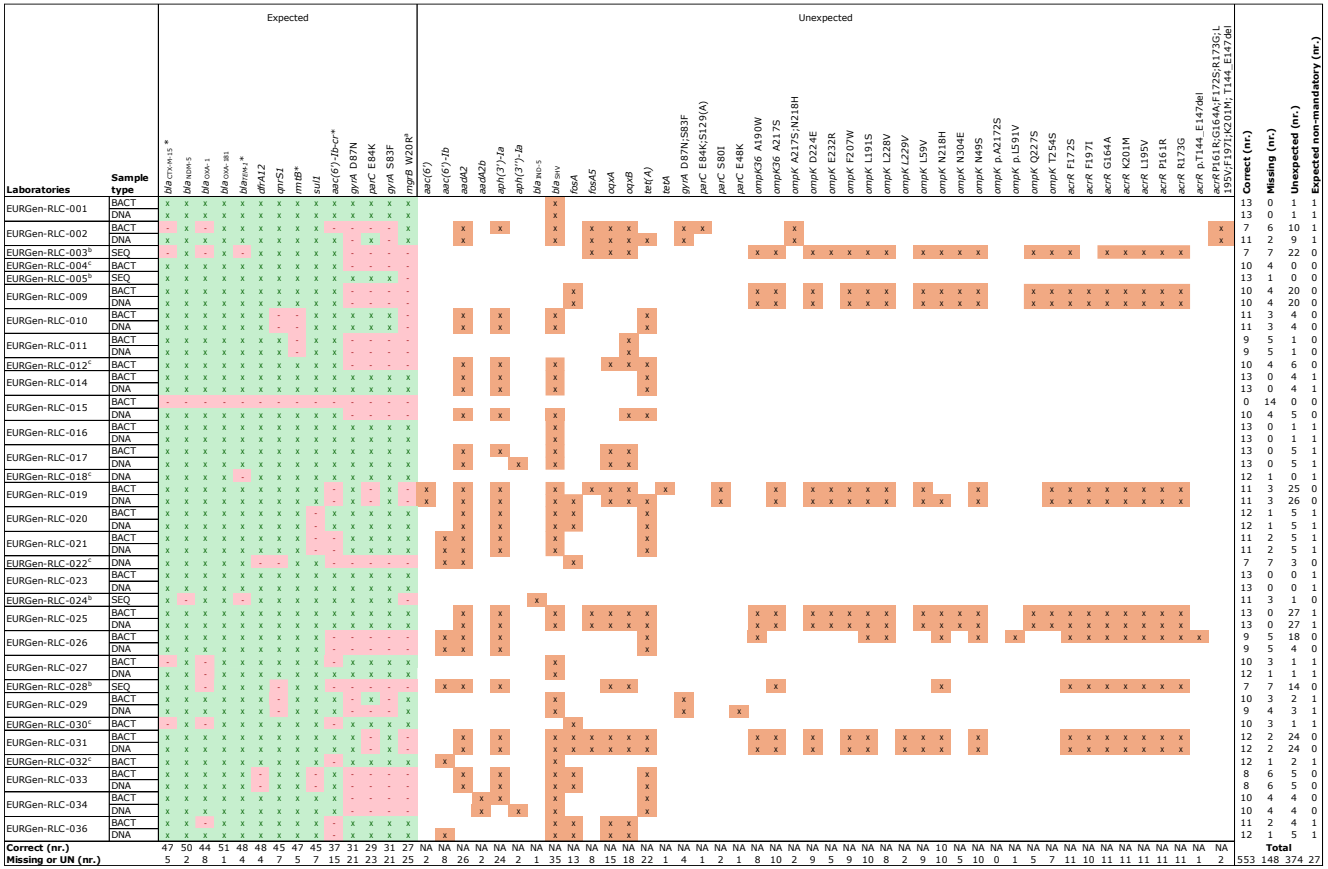
Table 21. Results of the detection of genetic AMR determinants for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-03 (*A. baumannii*)

Laboratories	Sample type	Expected: *ant(2'')-Ia*	*bla* OXA-23	*gyrA* S81L	*parC* S84L	*parC* V104I[a]	*parC* D105E[a]	Unexpected: *aadA2*	*aadA21*	*aadA2b*	*abaF*	*ant(3'')-Ia*	*ant(3'')-IIa*	*aph(3'')-Ib*	*aph(6)-Ib*	*aph(6)-Id*	*bla* ADC-25	*bla* ADC-262	*bla* CARB-2	*bla* OXA-429	*dfrA45*	*sul1*	*sul2*	*tet(B)*	*tet(G)*	*adeS* G336S	*gyrA* gyrA_S81L	*parC* parC_S84L	Correct (nr.)	Missing (nr.)	Unexpected (nr.)	Expected non-mandatory (nr.)
EURGen-RLC-001	BACT	x	x	x	x	-	-																						4	0	0	0
	DNA	x	x	x	x	-	-																						4	0	0	0
EURGen-RLC-003[b]	SEQ	x	x	-	-	-	-																						2	2	0	0
EURGen-RLC-004[c]	BACT	x	x	x	x	-	-																						4	0	0	0
EURGen-RLC-005[b]	SEQ	x	x	x	x	x	x																						4	0	0	2
EURGen-RLC-009	BACT	x	x	-	-	-	-																						2	2	0	0
	DNA	x	x	-	-	-	-																						2	2	0	0
EURGen-RLC-010	BACT	x	x	-	-	-	-	x					x	x				x	x	x	x			x	x				2	2	10	0
	DNA	x	x	-	-	-	-	x					x	x				x	x	x	x			x	x				2	2	10	0
EURGen-RLC-011	BACT	x	-	-	-	-	-																						1	3	0	0
	DNA	x	-	-	-	-	-																						1	3	0	0
EURGen-RLC-014	BACT	x	x	x	x	-	-	x			x		x	x				x	x	x	x			x	x				4	0	11	0
	DNA	x	x	x	x	-	-		x				x	x				x	x	x	x			x	x				4	0	10	0
EURGen-RLC-015[c]	BACT	x	x	x	x	-	-	x						x	x				x	x				x	x	x			4	0	9	0
	DNA	-	-	-	-	-	-																						0	4	0	0
EURGen-RLC-016	BACT	x	x	x	x	-	-																						4	0	0	0
	DNA	x	x	x	x	-	-																						4	0	0	0
EURGen-RLC-017	BACT	x	x	x	x	-	-	x					x	x		x		x	x		x								4	0	7	0
	DNA	x	x	x	x	-	-	x					x	x		x		x	x		x								4	0	7	0
EURGen-RLC-018[c]	DNA	x	x	x	x	-	-																						4	0	0	0
EURGen-RLC-020	BACT	x	x	x	x	-	-	x			x		x	x		x		x	x	x	x			x	x				4	0	11	0
	DNA	x	x	x	x	-	-	x			x	x		x		x		x	x	x	x			x	x				4	0	11	0
EURGen-RLC-021	BACT	x	x	x	x	-	-	x			x			x		x		x	x	x	x			x	x	x			4	0	11	0
	DNA	x	x	x	x	-	-	x			x			x		x		x	x	x	x			x	x	x			4	0	11	0
EURGen-RLC-022[c]	DNA	x	-	-	-	-	-	x											x	x		x		x	x				1	3	6	0
EURGen-RLC-023	BACT	x	x	-	-	-	-																						2	2	0	0
	DNA	x	x	-	-	-	-																						2	2	0	0
EURGen-RLC-024[b]	SEQ	x	x	x	x	x	x																						4	0	0	2
EURGen-RLC-025	BACT	x	x	x	x	-	-	x			x		x	x		x		x	x	x	x			x	x	x			4	0	13	0
	DNA	x	x	x	x	-	-	x			x		x	x		x		x	x	x	x			x	x	x			4	0	13	0
EURGen-RLC-026	BACT	x	x	-	-	-	-	x						x		x	x		x	x				x	x	x			2	2	9	0
	DNA	x	x	-	-	-	-	x						x		x	x		x	x				x	x	x			2	2	9	0
EURGen-RLC-028[b]	SEQ	x	x	x	x	x	x			x							x			x			x						4	0	5	2
EURGen-RLC-029	BACT	x	x	x	x	-	-											x	x	x									4	0	3	0
	DNA	x	x	x	x	-	-											x	x	x									4	0	3	0
EURGen-RLC-030[c]	BACT	x	x	x	x	-	-																						4	0	0	0
EURGen-RLC-031	BACT	x	x	-	-	-	-	x						x		x			x	x				x	x	x			2	2	8	0
	DNA	x	x	-	-	-	-	x						x		x			x	x				x	x	x			2	2	8	0
EURGen-RLC-032[c]	BACT	x	x	x	x	-	-																						4	0	0	0
EURGen-RLC-033	BACT	x	x	-	-	-	-	x			x		x	x		x		x	x	x				x	x		x	x	2	2	12	0
	DNA	x	x	-	-	-	-	x			x		x	x		x		x	x	x				x	x		x	x	2	2	12	0
EURGen-RLC-034	BACT	x	x	-	-	-	-			x									x				x						2	2	3	0
	DNA	x	x	-	-	-	-			x									x				x						2	2	3	0
EURGen-RLC-036	BACT	x	x	x	x	-	-													x									4	0	1	0
	DNA	x	x	x	x	-	-													x									4	0	1	0
EURGen-RLC-002	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-012	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-019	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-027	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
Correct (nr.)		44	41	26	26	3	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	**Total**			
Missing or UN (nr.)		1	4	19	19	42	42	19	1	3	9	1	11	19	1	14	3	16	25	23	12	1	14	18	18	5	2	2	137	43	217	6

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): Genetic AMR determinant reported
Cells shaded in red (-): Genetic AMR determinant missing
Cells shaded in orange (x): Unexpected genetic AMR determinant reported
Cells shaded in grey (/): participant did not submit AMR genes and mutations
[a] Expected results but non mandatory to report
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both
[d] The laboratory did not submit results for EURGen-2024-03

For strain EURGen-2024-04 (*K. pneumoniae*), participants were expected to detect 10 AMR genes (*aac(6')-Ib-cr* or *aac(6')-Ib-cr5* or *aac(6')-Ib-cr6, bla*$_{\text{CTX-M-15}}$ or *bla*$_{\text{CTX-M-101}}$, *bla*$_{\text{NDM-5}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-181}}$, *bla*$_{\text{TEM-1}}$or *bla*$_{\text{TEM-1A}}$ or *bla*$_{\text{TEM-1B}}$ or *bla*$_{\text{TEM-1C}}$ or *bla*$_{\text{TEM-1D}}$, *dfrA12, qnrS1, rmtB* or *rmtB1*, *sul1*) and three chromosomal PMs (*gyrA* D87N, *gyrA* S83F, *parC* E84K). One PM (*mgrB* W20R) was also expected but not mandatory to report. In total, 52 sets of results were submitted by 31 laboratories for the detection of AMR determinants for this strain. Of these, 25 sets of results were submitted for BACT while 23 were submitted for DNA samples. Two sets of submitted results contained all the expected AMR determinants without any unexpected determinant. The most reported AMR determinant in all sets of results was *bla*$_{\text{OXA-181}}$ (n=51), followed by *bla*$_{\text{NDM-5}}$ (n=50). The most frequently missed AMR determinant in all sets of results was the PM *parC* E84K (n=23), followed by PMs *gyrA* D87N and *gyrA* S83F missing in 21 sets of results each. There were 59 other cases of other missing expected genetic determinants from submitted results. The expected non-mandatory PM *mgrB* W20R was missing in 25 sets of results. The number of unexpected genetic determinants of AMR reported by the participants was 374. There were 17 different unexpected PMs in *ompK* and nine in *acrR*. The most reported unexpected AMR gene was *bla*$_{\text{SHV}}$ (n=35), followed by *aadA2* (n=26), *aph(3')-Ia* (n=24), *tet(A)* (n=22), *oqxB* (n=18) and *oqxA* (n=15) (Table 22).

Table 22. Results of the detection of genetic AMR determinants for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-04 (*K. pneumoniae*)



NA: Not applicable; UN: Unexpected
Cells shaded in green (x): Genetic AMR determinant reported
Cells shaded in red (-): Genetic AMR determinant missing
Cells shaded in orange (x): Unexpected genetic AMR determinant reported
NA: Not applicable; UN: Unexpected
[a] Expected results but non-mandatory to report
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both
[d] The laboratory did not submit results for EURGen-2024-04
* Either *bla*$_{CTX-M-15}$ or *bla*$_{CTX-M-101}$, either *bla*$_{TEM-1}$ or *bla*$_{TEM-1A}$ or *bla*$_{TEM-1B}$ or *bla*$_{TEM-1C}$ or *bla*$_{TEM-1D}$, either *aac(6')-Ib-cr* or *aac(6')-Ib-cr5* or *aac(6')-Ib-cr6, either rmtB* or *rmtB1*

More than 70% of submitted sets of results were incomplete and were missing one or more expected genetic AMR determinants. Most of these missing determinants were chromosomal PMs associated with AMR. This is likely due to the lack of species-specific chromosomal PMs databases in some of the bioinformatics tools. For example, the integrated PMs database (PointFinder) in the ResFinder tool does not contain PMs for *A. baumannii* and *P. aeruginosa*. For generating the expected results for these species, consensus results from AMRFinderPlus (with AMRFinderPlus database) and RGI (with CARD database) were used for the PMs and most participants that used either of these databases were able to detect the expected PMs in *A. baumannii* and *P. aeruginosa.* Surprisingly, there were still several laboratories that used either CARD or AMRFinderPlus databases and did not report the expected PMs in those species. This might be due to the use of local pipelines and algorithms by these laboratories for the detection of AMR determinants, or due to the use of different versions of the databases than those used to prepare the expected results. To solve these limitations, best practices include ensuring that databases integrated into local pipelines are constantly updated and furthermore confirming that the databases in use target the species under analysis.

Many laboratories submitted the expected PMs but did not follow the instructions described in the 2024 EQA protocol for submission of PMs in the webtool, hence these PMs did not match the accepted formats and were automatically scored as unexpected. To accommodate mutations other than silent, missense or nonsense PMs, such as indels and gene disruptions caused by insertion elements, the EQA webtool allowed the reporting of mutations without any limitation in the text field, unlike the previous EQA webtools where only PMs with a specific format were allowed. Participants received specific instructions on how to format, with specific examples. However, a large number of PMs were reported in other formats. For example, two laboratories reported PM *gyrA* p.D87Y instead of *gyrA* D87Y for EURGen-2024-01. Other participants reported *parC* parC_S80I and *uhpT* uhpT_E350Q instead of *parC* S80I and *uhpT* E350Q, respectively. Moreover, one laboratory reported two PMs in *gyrA* (gyrA D87Y and *gyrA* S83L) in the same field (*gyrA* D87Y, S83L), contrary to the 2024 EQA protocol where it was instructed to submit only one mutation per field. It should be noted that these mutations were detected while preparing the expected results and the discordances were not due to the lack of bioinformatics capacity of the laboratories to detect these mutations, rather a result of not following the guidelines in the EQA protocol for submission of PMs in the 2024 EQA webtool. It is important that laboratories strengthen their good practices and ensure that they report of bioinformatics results following the specific instructions and frameworks of the reporting systems, because often the information is further processed automatically and fields that do not match the accepted formats are not considered for later analysis.

Similarly, the *glpT* E448K and *uhpT* E350Q conferring resistance to fosfomycin were the most frequently missed PMs in strain EURGen-2024-01 (*E. coli*). These mutations are absent in ResFinder (PointFinder database) but are present in AMRFinderPlus and CARD databases. Therefore, the laboratories that only used ResFinder for detecting PMs conferring AMR were not able to detect these mutations. Resistance against an antimicrobial agent is sometimes due to the combination of multiple resistance mechanisms, and multiple chromosomal PMs may have a cumulative effect, thus it is especially important to obtain a complete profile of these mutations. Therefore, participants may consider using confirmatory bioinformatics tools and databases for the detection of AMR determinants to obtain a complete genetic AMR profile. Moreover, there were laboratories which used AMRFinderPlus and RGI (with CARD database) but still did not report these mutations for the strain, possibly due to the use of different versions of the databases than those used to prepare the expected results or lack of knowledge regarding the impact of the mutations on resistance profiles.

In the current EQA, most of the discrepancies that exist in the databases were considered while preparing the expected results. For example, the aminoglycoside resistance genes *aac(6')-Ib*, *aac(6')-Ib-Hangzhou*, *aac(6')-Ib3*, *aac(6')-Ib4* and *aac(6')-Ib9* have a high sequence similarity between them (>99%) and depending on the bioinformatics tools used, any of these genes might be detected and reported by the participants. Similarly, the gene with same sequence is named *ant(3'')-Ii-aac(6')-Iid* in CARD and *ant(3'')-Ih/aac(6')-IId* in AMRFinderPlus. Another example of discrepancy between databases is that ResFinder reports aminoglycoside resistance gene *aac(6')-Ib-cr* while CARD and AMRFinderPlus report the same region as *aac(6')-Ib-cr5 or aac(6')-Ib-cr6*. To compensate for these discrepancies and to ensure correct scoring, the webtool in 2024 EQA was designed to accept any of these genes as correct answers. Surprisingly, 19 sets of results were missing any of the accepted variant of *aac(6')-Ib* for strain EURGen2024-02 (*P. aeruginosa*).

The most frequently missing AMR genes belonged to the group of aminoglycoside resistance genes (missed 64 times). Specifically, *aac(6')-Ib* (or *aac(6')-Ib-Hangzhou* or *aac(6')-Ib3* or *aac(6')-Ib4* or *aac(6')-Ib9*) was missed 19 times, while *aph(3')-VI* (or *aph(3')-VIa*) was missed 13 times in strain EURGen-2024-02 (*P. aeruginosa*). Similarly, *aac(6')-Ib-cr* (or *aac(6')-Ib-cr5* or *aac(6')-Ib-cr6*) was missed 15 times while *rmtB* (or *rmtB1*) was missed five times in strain EURGen-2024-04 (*K. pneumoniae*). Moreover, genes encoding β-lactamases were missed 45 times. Specifically, *bla*$_{\text{PME-1}}$ in strain EURGen-2024-02 was missed 11 times, while *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{CTX-M-15}}$ (or *bla*$_{\text{CTX-M-101}}$) in strain EURGen-2024-04 were missed eight and five times each. Furthermore, the genes encoding the enzymes that inhibit folate synthesis were missed 49 times (*dfrA12* (n=11); *dfrA17* (n=6); *sul1* (n=15); *sul2* (n=8); *sul3* (n=9)), while quinolone resistance genes were missed 28 times (*crpP* (n=18); *qnrS1* (n=7); *qnrVC1* (n=3)).

Only a few participants reported non-mandatory expected AMR determinants. These determinants were classified as non-mandatory because while preparing the expected results for the 2024 EQA there was no consensus between sets of results obtained from different bioinformatics tools or using different types of sequence data. For example, in EURGen-2024-02 (*P. aeruginosa*) the chromosomal PM *parC* S87L, associated with decreased susceptibility towards quinolones, was only detected in AMRFinderPlus. Similarly, *nalC* mutations G71E and S209R that contribute to increased resistance to quinolones were only detected with RGI (CARD database). The non-mandatory expected PMs *parC* V104I and *parC* D105E for EURGen-2024-03 (*A. baumannii*), that confer high-level quinolone resistance in the species, were only detected with RGI (CARD database) and were only reported by three participants. The PM *mgrB* W20R in EURGen-2024-04 (*K. pneumoniae*) was only detected with AMRFinderPlus and most of the participants failed to detect it (n=25 times).

These problems suggest that a better harmonization between bioinformatics tools and their respective databases is needed, to ensure that the same genetic sequences have the same designation across databases and the same potential for being detected across tools. A solution could be to ensure communication between curators of the most widely used databases, and to opt to use sequences and nomenclature that are part of reference sequence databases such as NCBI RefSeq. Also, consolidation and synchronization of the databases before the release of new database versions might be helpful to eliminate these discrepancies. Furthermore, these findings support that the proper recording of bioinformatics tools, their respective versions and date of analysis are of paramount importance to allow for validation, traceability, and comparison of results within and between settings. It is important that the users of these tools and databases report any discrepancies directly to the curators.

Additionally, in some of the situations described previously, and others, there were presumable spelling, distraction, or submission mistakes, such as submission of *glpT* T448K instead of expected PM *glpT* E448K for strain EURGen-2024-01 (*E. coli*). Another example was the submission of PM *gyrA* D97Y instead of expected PM *gyrA* D87Y for strain EURGen-2024-01. The reporting of PMs *gyrA* S87L and *parC* T83I instead of the expected PMs *gyrA* T83I and *parC* S87L, respectively, in EURGen-2024-02 (*P. aeruginosa*) were seemingly due to distractions while submitted results into the webtool. It is important to report the AMR gene or PM correctly and carefully, since reporting an unexpected genetic determinant can affect the prediction of antimicrobial susceptibility profile and considering that many of the analyses can be performed automatically by algorithms that cannot recognise spelling variations. Therefore, participants should ensure more attentive review, recording and reporting of results while working with these data.

In addition to the missing AMR determinants, another common issue was the reporting of unexpected genetic AMR determinants by the participants, with 73% of sets of results containing unexpected AMR determinants. In some situations, this was due to insufficient knowledge regarding the impact of certain genes or PMs in the expected resistance profiles of the species included in this EQA. Some participants have reported genes for species in which they are intrinsic and do not contribute to the elevated resistance. For example, several laboratories reported *fosA* (n=25 times), *bla*$_{\text{CARB-2}}$ (n=23 times), *aph(3')-IIb* (n=18 times), *bla*$_{\text{OXA-486}}$ (n=18 times), *bla*$_{\text{PDC-5}}$ (n=17 times), and *bla*$_{\text{PAO}}$ (n=10 times) in strain EURGen-2024-02 (*P. aeruginosa*). Although these genes are present in this strain, they are intrinsic in *P. aeruginosa* and do not contribute to a decrease in the susceptibility to the respective antimicrobials included in this EQA. Moreover, intrinsic resistance genes were incorrectly reported for strain EURGen-2024-03 (*A. baumannii*), specifically *bla*$_{\text{CARB-2}}$ (n=25 times), *bla*$_{\text{OXA-429}}$ (n=23 times) and *bla*$_{\text{ADC-25}}$ (n=16 times). For strain EURGen-2024-04 (*K. pneumoniae*), intrinsic resistance genes were also frequently reported. These included the *bla*$_{\text{SHV}}$ variants (n=35 times), *fosA* or *fosA5* (n=21 times), and multidrug resistance efflux pump genes *oqxA* and/or *oqxB* (n=33 times), which are intrinsic in *K. pneumoniae*. In the previous EQAs, *bla*$_{\text{SHV}}$ was considered as an acquired resistance gene in *K. pneumoniae,* however, new evidence suggests that the gene is intrinsic in the species[33]. Moreover, in some cases, participants reported chromosomal PMs in *gyrA*, *parC*, *and parE* that, albeit present, have not been proven to be associated with decreased susceptibility towards quinolones. Similarly, several mutations were also reported in the target genes *acrR, adeS, cyaA,* and *ompK*, for which experimental evidence of the impact on AMR profiles of these strains is lacking.

Most of the remaining unexpectedly reported genetic determinants seemed due to the misinterpretation of the 2024 EQA protocol or incomplete knowledge regarding the genetic mechanisms of AMR. Some participants have frequently reported acquired AMR genes and PMs which are present in the EQA test strains, however the antimicrobials that they confer resistance towards were not part of the 2024 EQA. For example, participants reported genes conferring resistance to antimicrobials not included in this EQA such as tetracycline (*tet(A), tet(B), tet(G), tet(M)*), streptomycin (*aadA1, aadA2, aadA2b, aadA5, aadA10, aadA11, aadA15, aadA21, ant(3'')-Ia, ant(3'')-IIa, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id*) and kanamycin (*aph(3')-IIb*). Seemingly, several participants reported all genetic determinants detected by the bioinformatics tools without carefully examining the data or reporting instructions including reporting different variants for the same genetic location. Most bioinformatics tools include the information about the location of the gene on the sequence being analysed, (the information about the contig number, start and end locations within that contig, and size of the gene) and participants are encouraged to confirm the genomic location of the relevant genetic determinants when analysing sequence data, including flanking regions, to confirm if different variants are present

[33] https://doi.org/10.1016/j.diagmicrobio.2017.01.005

simultaneously or if this is an artifact of the bioinformatics tools.

These situations emphasize that laboratories should take a judicious and critical approach while reporting the genetic determinants of AMR and become familiar with the underlying genetic mechanisms of resistance that are relevant for the different species analysed in their settings. Furthermore, very important information can become more difficult to retrieve in the midst of very large datasets of results and make it challenging to reach clinically and epidemiologically relevant conclusions.

3.7. *In silico* prediction of antimicrobial resistance profiles

In silico prediction of AMR profiles was generally conducted simultaneously with the detection of the genetic determinants mediating AMR (Appendix 5).

Overall, 28 participants submitted results for the *in silico* prediction of AMR profiles. Three laboratories did not submit results for any sample. Nineteen laboratories submitted the AMR profiles for both BACT and DNA samples while five laboratories submitted AMR profiles for either BACT or DNA samples. Four laboratories submitted AMR profiles using the sequences provided by the EQA organizer.

In total, 173 AMR profiles were submitted for all the strains, and 23.7% were fully correct (n=41). Additionally, in 31.8% of the submitted AMR profiles (n=55) certain expected antimicrobials were missing. Moreover, in 12.1% of the submitted AMR profiles (n=21), unexpected antimicrobials that were not part of the expected AMR profiles were reported. In 56% of the submitted results, certain antimicrobials were missing and simultaneously contained unexpected antimicrobials (n=56) (Table 23, Figure 9).

Table 23. Distribution of submitted results regarding the *in silico* prediction of AMR profiles in the EURGen-RefLabCap 2024 EQA

Test strain	Correct profiles	Only missing antimicrobials	Only unexpected antimicrobials	Missing and Un-expected antimicrobials	Total
EURGen-2024-01 (*E. coli*)	17	26	0	4	**47**
EURGen-2024-02 (*P. aeruginosa*)	5	15	2	17	**39**
EURGen-2024-03 (*A. baumannii)*	5	12	12	12	**41**
EURGen-2024-04 (*K. pneumoniae*)	14	2	7	23	**46**
Total	**41**	**55**	**21**	**56**	**173**

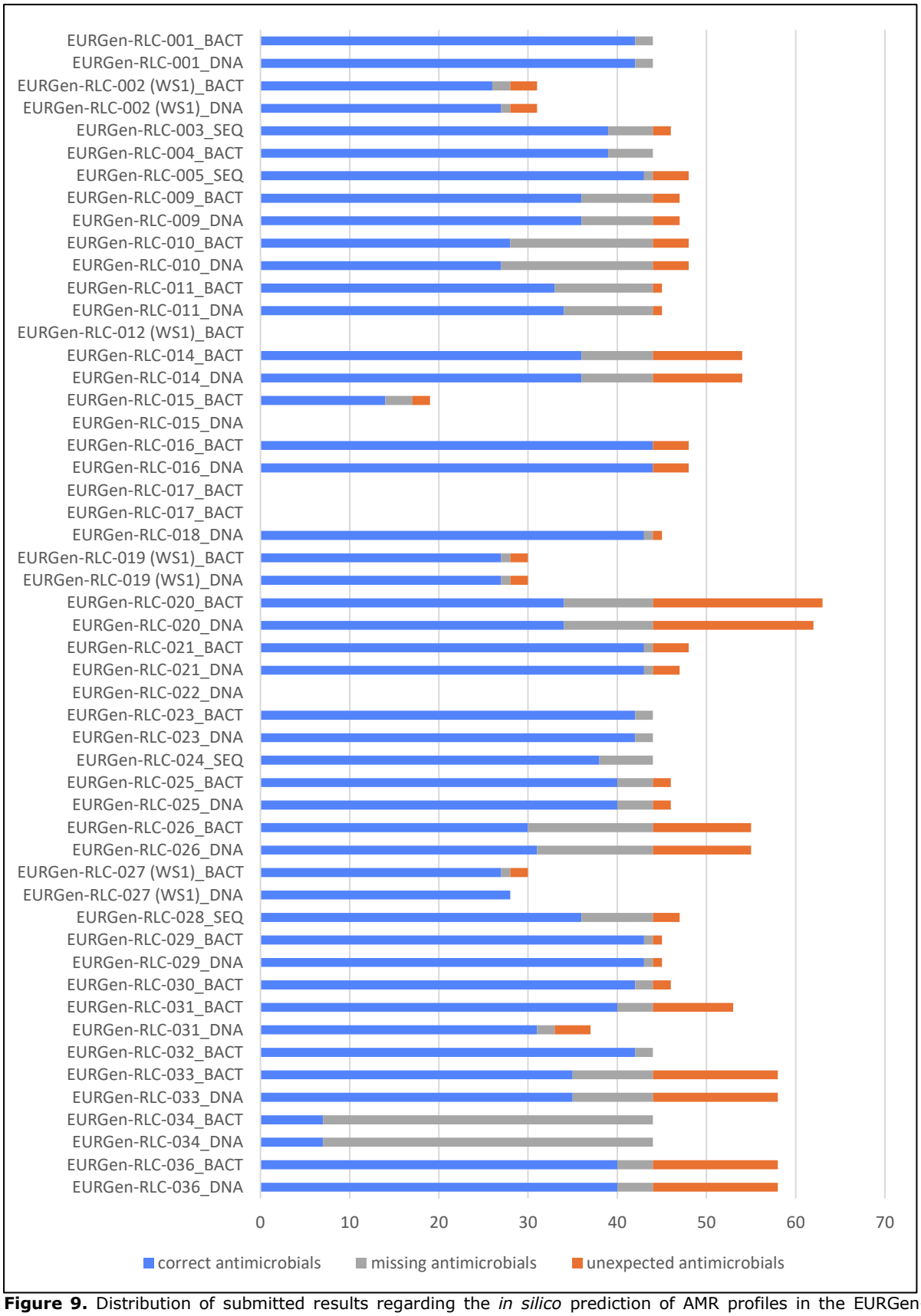


Figure 9. Distribution of submitted results regarding the *in silico* prediction of AMR profiles in the EURGen-RefLabCap 2024 EQA, for each participant and for each type of dataset (BACT, DNA or SEQ). WS1: Participants only analysed material belonging to the WS1 (*E. coli* and *K. pneumoniae*)

Overall, the participants obtained points for the prediction of AMR profiles which corresponded to 15.9% to 100% of their maximum possible scores for the expected antimicrobials (Table 13, Figure 5). The average concordance between expected and submitted antimicrobials was 85.2%. For the BACT samples, none of the participants correctly predicted AMR profiles for all test strains analysed. For DNA samples, only one laboratory correctly predicted the AMR profile of all the analysed strains.

For strain EURGen-2024-01 (*E. coli*), participants were expected to predict resistance towards 12 antimicrobials (ampicillin, aztreonam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, colistin, fosfomycin, gentamicin, sulfamethoxazole, tobramycin, trimethoprim). In total, 29 laboratories submitted 47 AMR profiles for this strain. Among 47 AMR profiles, 23 were submitted for BACT while 20 were submitted for DNA samples. The expected antimicrobial colistin was reported in all 47 AMR profiles. The most frequent expected antimicrobial missing from the expected AMR profiles was aztreonam (n=18), followed by ciprofloxacin (n=10) and tobramycin (n=9). The expected antimicrobials ampicillin and sulfamethoxazole were not reported by seven participants each. The total number of missing antimicrobials throughout all submitted results was 67. Overall, 17 AMR profiles contained all expected antimicrobials without any unexpected antimicrobial. In 26 AMR profiles, at least one expected antimicrobial was missing. All participants reported at least one expected antimicrobial. One laboratory missed all antimicrobials except colistin for both BACT and DNA samples. Overall, three unexpected antimicrobials were reported and included amikacin, ertapenem and tigecycline, reported in two AMR profiles each, for a total of six unexpected antimicrobials reported by the participants. The complete description of the concordances and discordances between the expected AMR profiles and the results submitted by participants is provided in Table 24.

Table 24. Results of the *in silico* prediction of AMR profiles for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-01 (*E. coli*)

Laboratories	Sample type	Expected: Ampicillin	Aztreonam	Cefepime	Cefotaxime	Ceftazidime	Ciprofloxacin	Colistin	Fosfomycin	Gentamicin	Sulfamethoxazole	Tobramycin	Trimethoprim	Unexpected: Amikacin	Ertapenem	Tigecycline	Correct (nr.)	Missing (nr.)	Unexpected (nr.)
EURGen-RLC-001	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-002	BACT	x	x	x	x	-	x	x	x	x	x	x	x				11	1	0
	DNA	x	x	x	x	-	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-003[b]	SEQ	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-004[c]	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-005[b]	SEQ	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-009	BACT	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
	DNA	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-010	BACT	x	x	x	x	x	-	x	x	x	x	x	x				11	1	0
	DNA	x	x	x	x	x	-	x	x	x	x	x	x				11	1	0
EURGen-RLC-011	BACT	x	-	x	x	x	-	x	x	x	x	x	x				10	2	0
	DNA	x	-	x	x	x	-	x	x	x	x	x	x				10	2	0
EURGen-RLC-014	BACT	-	-	x	x	x	x	x	x	x	x	x	x				10	2	0
	DNA	-	-	x	x	x	x	x	x	x	x	x	x				10	2	0
EURGen-RLC-015	BACT	x	-	x	x	x	x	x	x	x	-	x	x				10	2	0
EURGen-RLC-016	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-018[c]	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-019	BACT	x	x	x	x	x	-	x	x	x	x	x	x				11	1	0
	DNA	x	x	x	x	x	-	x	x	x	x	x	x				11	1	0
EURGen-RLC-020	BACT	x	x	x	x	x	x	x	x	x	-	-	x		x	x	10	2	2
	DNA	x	x	x	x	x	x	x	x	x	-	-	x		x	x	10	2	2
EURGen-RLC-021	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-023	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-024[b]	SEQ	x	x	x	x	x	x	x	x	-	x	-	x				10	2	0
EURGen-RLC-025	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-026	BACT	-	-	x	x	x	-	x	x	-	x	-	x				7	5	0
	DNA	-	-	x	x	x	-	x	x	x	x	-	x				8	4	0
EURGen-RLC-027	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-028[b]	SEQ	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-029	BACT	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
	DNA	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-030[c]	BACT	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-031	BACT	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x				12	0	0
EURGen-RLC-032[c]	BACT	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-033	BACT	x	x	x	x	x	x	x	x	x	-	-	x	x			10	2	1
	DNA	x	x	x	x	x	x	x	x	x	-	-	x	x			10	2	1
EURGen-RLC-034	BACT	-	-	-	-	-	-	x	-	-	-	-	-				1	11	0
	DNA	-	-	-	-	-	-	x	-	-	-	-	-				1	11	0
EURGen-RLC-036	BACT	x	-	x	x	x	x	x	x	x	x	x	x				11	1	0
	DNA	-	x	x	x	x	x	x	x	x	x	x	x				11	1	0
EURGen-RLC-012[c]	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA
EURGen-RLC-015	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA
EURGen-RLC-017	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA
EURGen-RLC-022[c]	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA
Correct (nr.)		40	29	45	45	43	37	47	45	43	40	38	45	NA	NA	NA	**Total**		
Missing or UN (nr.)		7	18	2	2	4	10	0	2	4	7	9	2	2	2	2	497	67	6

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): AMR profile reported for the antimicrobial
Cells shaded in red (-): AMR profile missing for the antimicrobial
Cells shaded in orange (x): AMR profile reported for the unexpected antimicrobial
Cells shaded in grey (/): participant did not submit AMR profile
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both

For strain EURGen-2024-02 (*P. aeruginosa*), participants were expected to predict resistance towards 11 antimicrobials (amikacin, aztreonam, cefepime, ceftazidime, ceftazidime-avibactam, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin-tazobactam, tobramycin). In total, 24 laboratories submitted the 39 AMR profiles for this strain. Among 39 AMR profiles, 19 were submitted for BACT while 16 were submitted for DNA samples. The most frequent expected antimicrobial missing from the AMR profiles of this strain was aztreonam (n=27), followed by ceftazidime-avibactam (n=22), and tobramycin (n=19). The expected antimicrobials amikacin and gentamicin were missing in 13 AMR profiles each. There were 28 more cases of other missing antimicrobials. There were 122 total instances where expected antimicrobials were missing. In total, there were 78 unexpected antimicrobials reported. The most reported unexpected antimicrobial for this strain was fosfomycin (n=20). Overall, five AMR profiles contained all expected antimicrobials without any unexpected antimicrobial. All participants reported at least one expected antimicrobial. The complete description of the results submitted by participants is provided in Table 25.

Table 25. Results of the *in silico* prediction of AMR profiles for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-02 (*P. aeruginosa*)

		Expected											Unexpected									
Laboratories	**Sample type**	Amikacin	Aztreonam	Cefepime	Ceftazidime	Ceftazidime-avibactam	Ciprofloxacin	Gentamicin	Imipenem	Meropenem	Piperacillin-tazobactam	Tobramycin	Amoxicillin-clavulanic acid	Ampicillin	Cefotaxime	Ertapenem	Fosfomycin	Sulfamethoxazole	Tigecycline	**Correct (nr.)**	**Missing (nr.)**	**Unexpected (nr.)**
EURGen-RLC-001	BACT	x	-	x	x	x	x	-	x	x	x	x								9	2	0
	DNA	x	-	x	x	x	x	-	x	x	x	x								9	2	0
EURGen-RLC-003[b]	SEQ	x	-	x	x	-	x	x	x	x	x	x					x			9	2	1
EURGen-RLC-004[c]	BACT	-	-	x	x	x	x	-	x	x	x	-								7	4	0
EURGen-RLC-005[b]	SEQ	x	-	x	x	x	x	x	x	x	x	x								10	1	0
EURGen-RLC-009	BACT	-	-	x	x	-	x	-	x	x	x	-					x			6	5	1
	DNA	-	-	x	x	-	x	-	x	x	x	-					x			6	5	1
EURGen-RLC-010	BACT	x	-	-	-	-	-	x	-	-	-	-					x		x	2	9	2
	DNA	x	-	-	-	-	-	x	-	-	-	-					x		x	2	9	2
EURGen-RLC-011	BACT	-	-	x	x	-	x	-	x	x	x	-								6	5	0
	DNA	-	-	x	x	-	x	-	x	x	x	-								6	5	0
EURGen-RLC-014	BACT	-	-	x	x	x	-	-	x	x	x	-	x	x	x	x	x		x	6	5	6
	DNA	-	-	x	x	x	-	-	x	x	x	-	x	x	x	x	x		x	6	5	6
EURGen-RLC-016	BACT	x	x	x	x	x	x	x	x	x	x	x								11	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x								11	0	0
EURGen-RLC-018[c]	DNA	x	-	x	x	x	x	x	x	x	x	x								10	1	0
EURGen-RLC-020	BACT	x	x	x	x	-	-	x	x	x	-	-		x	x	x	x		x	7	4	5
	DNA	x	x	x	x	-	-	x	x	x	-	-		x	x	x	x		x	7	4	5
EURGen-RLC-021	BACT	x	x	x	x	x	x	x	x	x	x	x					x			11	0	1
	DNA	x	x	x	x	x	x	x	x	x	x	x					x			11	0	1
EURGen-RLC-023	BACT	x	-	x	x	x	x	x	x	x	x	x								10	1	0
	DNA	x	-	x	x	x	x	x	x	x	x	x								10	1	0
EURGen-RLC-024[b]	SEQ	-	x	x	x	-	x	-	x	x	x	-								7	4	0
EURGen-RLC-025	BACT	x	-	x	x	-	x	x	x	x	x	x					x			9	2	1
	DNA	x	-	x	x	-	x	x	x	x	x	x					x			9	2	1
EURGen-RLC-026	BACT	-	-	x	x	-	x	x	x	x	x	-	x	x	x	x	x	x	x	7	4	7
	DNA	-	-	x	x	-	x	x	x	x	x	-	x	x	x	x	x	x	x	7	4	7
EURGen-RLC-028[b]	SEQ	-	-	x	x	-	x	-	x	x	x	-								6	5	0
EURGen-RLC-029	BACT	x	x	x	x	x	x	x	x	x	x	x								11	0	0
	DNA	x	x	x	x	x	x	x	x	x	x	x								11	0	0
EURGen-RLC-030[c]	BACT	x	x	x	x	x	x	x	x	x	x	x								11	0	0
EURGen-RLC-031	BACT	x	-	x	x	-	x	x	x	x	x	x	x	x	x	x	x			9	2	5
EURGen-RLC-032[c]	BACT	x	-	x	x	x	x	x	x	x	x	x								10	1	0
EURGen-RLC-033	BACT	x	x	x	x	-	x	x	x	x	-	-	x	x	x	x	x		x	8	3	6
	DNA	x	x	x	x	-	x	x	x	x	-	-	x	x	x	x	x		x	8	3	6
EURGen-RLC-034	BACT	-	-	-	-	-	-	-	x	x	-	-								2	9	0
	DNA	-	-	-	-	-	-	-	x	x	-	-								2	9	0
EURGen-RLC-036	BACT	x	-	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	9	2	7
	DNA	x	-	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	9	2	7
EURGen-RLC-002	BACT																			NA	NA	NA
	DNA																			NA	NA	NA
EURGen-RLC-012[c]	BACT																			NA	NA	NA
EURGen-RLC-015	BACT																			NA	NA	NA
	DNA																			NA	NA	NA
EURGen-RLC-017	BACT																			NA	NA	NA
	DNA																			NA	NA	NA
EURGen-RLC-019	BACT																			NA	NA	NA
	DNA																			NA	NA	NA
EURGen-RLC-022[c]	DNA																			NA	NA	NA
EURGen-RLC-027	BACT																			NA	NA	NA
	DNA																			NA	NA	NA
EURGen-RLC-031	DNA																			NA	NA	NA
Correct (nr.)		26	12	35	35	17	31	26	37	37	31	20	NA	NA	NA	NA	NA	NA	NA		**Total**	
Missing or UN (nr.)		13	27	4	4	22	8	13	2	2	8	19	9	11	11	11	20	4	12	307	122	78

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): AMR profile reported for the antimicrobial
Cells shaded in red (-): AMR profile missing for the antimicrobial
Cells shaded in orange (x): AMR profile reported for the unexpected antimicrobial
Cells shaded in grey (/): participant did not submit AMR profile
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both

For strain EURGen-2024-03 (*A. baumannii*), participants were expected to predict resistance towards five antimicrobials (ciprofloxacin, gentamicin, imipenem, meropenem, tobramycin). In total, 25 laboratories submitted 41 AMR profiles for this strain. Of these, 20 AMR profiles were submitted for BACT while 17 were submitted for DNA samples. In total, there were 36 cases of missing expected antimicrobials from the AMR profiles and the expected antimicrobial ciprofloxacin was missing in most of the AMR profiles (n=23). There were five AMR profiles that contained all the expected antimicrobials without any unexpected antimicrobial. In total, 72 unexpected antimicrobials were reported by the participants, including aztreonam (n=2) and fosfomycin (n=6) to which *A. baumannii* is intrinsically resistant. The most reported unexpected antimicrobial was ampicillin (n=14), followed by sulfamethoxazole (n=10). The complete description of the results submitted by participants is provided in Table 26.

Table 26. Results of the *in silico* prediction of AMR profiles for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-03 (*A. baumannii*)

		Expected					Unexpected																			
Laboratories	**Sample type**	Ciprofloxacin	Gentamicin	Imipenem	Meropenem	Tobramycin	Amoxicillin-clavulanic acid	Ampicillin	Aztreonam[d]	Cefepime	Cefotaxime	Ceftazidime	Ceftazidime-avibactam	Colistin	Ertapenem	Fosfomycin[d]	Piperacillin-tazobactam	Sulfamethoxazole	Tigecycline	Trimethoprim	**Correct (nr.)**	**Missing (nr.)**	**Unexpected (nr.)**
EURGen-RLC-001	BACT	x	x	x	x	x															5	0	0
	DNA	x	x	x	x	x															5	0	0
EURGen-RLC-003[b]	SEQ	-	x	x	x	x															4	1	0
EURGen-RLC-004[c]	BACT	x	-	x	x	x															4	1	0
EURGen-RLC-005[b]	SEQ	x	x	x	x	x				x		x	x				x				5	0	4
EURGen-RLC-009	BACT	-	x	x	x	x															4	1	0
	DNA	-	x	x	x	x															4	1	0
EURGen-RLC-010	BACT	-	x	-	-	x														x	2	3	1
	DNA	-	x	-	-	x														x	2	3	1
EURGen-RLC-011	BACT	-	-	x	x	x															3	2	0
	DNA	-	-	x	x	x															3	2	0
EURGen-RLC-014	BACT	-	x	x	x	x		x										x			4	1	2
	DNA	-	x	x	x	x		x										x			4	1	2
EURGen-RLC-015	BACT	-	x	x	x	x		x										x			4	1	2
EURGen-RLC-016	BACT	x	x	x	x	x				x		x	x				x				5	0	4
	DNA	x	x	x	x	x				x		x	x				x				5	0	4
EURGen-RLC-018[c]	DNA	x	x	x	x	x							x								5	0	1
EURGen-RLC-020	BACT	x	x	x	x	x		x	x	x	x	x			x	x			x	x	5	0	9
	DNA	x	x	x	x	x		x	x	x	x	x			x	x			x		5	0	8
EURGen-RLC-021	BACT	-	x	x	x	x										x					4	1	1
	DNA	-	x	x	x	x										x					4	1	1
EURGen-RLC-023	BACT	-	x	x	x	x															4	1	0
	DNA	-	x	x	x	x															4	1	0
EURGen-RLC-024[b]	SEQ	x	x	x	x	x															5	0	0
EURGen-RLC-025	BACT	-	x	x	x	x															4	1	0
	DNA	-	x	x	x	x															4	1	0
EURGen-RLC-026	BACT	-	x	x	x	-		x										x			3	2	2
	DNA	-	x	x	x	-		x										x			3	2	2
EURGen-RLC-028[b]	SEQ	-	x	x	x	x		x										x			4	1	2
EURGen-RLC-029	BACT	x	x	x	x	x								x							5	0	1
	DNA	x	x	x	x	x								x							5	0	1
EURGen-RLC-030[c]	BACT	x	x	x	x	x															5	0	0
EURGen-RLC-031	BACT	-	x	x	x	x		x										x			4	1	2
	DNA	-	x	x	x	x		x										x			4	1	2
EURGen-RLC-032[c]	BACT	x	x	x	x	x															5	0	0
EURGen-RLC-033	BACT	x	x	x	x	x	x	x							x	x				x	5	0	5
	DNA	x	x	x	x	x	x	x							x	x				x	5	0	5
EURGen-RLC-034	BACT	-	-	x	x	-															2	3	0
	DNA	-	-	x	x	-															2	3	0
EURGen-RLC-036	BACT	x	x	x	x	x	x	x							x		x	x			5	0	5
	DNA	x	x	x	x	x	x	x							x		x	x			5	0	5
EURGen-RLC-002	BACT																				NA	NA	NA
	DNA																				NA	NA	NA
EURGen-RLC-012[c]	BACT																				NA	NA	NA
EURGen-RLC-015	DNA																				NA	NA	NA
EURGen-RLC-017	BACT																				NA	NA	NA
	DNA																				NA	NA	NA
EURGen-RLC-019	BACT																				NA	NA	NA
	DNA																				NA	NA	NA
EURGen-RLC-022[c]	DNA																				NA	NA	NA
EURGen-RLC-027	BACT																				NA	NA	NA
	DNA																				NA	NA	NA
Correct (nr.)		18	36	39	39	37	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		**Total**	
Missing or UN (nr.)		23	5	2	2	4	4	14	2	5	2	5	4	2	6	6	5	10	2	5	169	36	72

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): AMR profile reported for the antimicrobial
Cells shaded in red (-): AMR profile missing for the antimicrobial
Cells shaded in orange (x): AMR profile reported for the unexpected antimicrobial
Cells shaded in grey (/): participant did not submit AMR profile
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both
[d] Intrinsic resistance (based on EUCAST Expected Phenotypes Version 1.2, January 2023)

For strain EURGen-2024-04 (*K. pneumoniae*), participants were expected to predict resistance towards 16 antimicrobials (amikacin, amoxicillin-clavulanic acid, aztreonam, cefepime, cefotaxime, ceftazidime, ceftazidime-avibactam, ciprofloxacin, ertapenem, gentamicin, imipenem, meropenem, piperacillin-tazobactam, sulfamethoxazole, tobramycin, trimethoprim). Colistin was expected in the AMR profile of this strain but was non-mandatory to report (accepted as a correct result but not a requirement for a fully correct AMR profile). In total, 27 laboratories submitted 46 AMR profiles for this strain. Of these, 22 AMR profiles were submitted for BACT while 20 were submitted for DNA samples. The expected antimicrobials imipenem and meropenem were reported by all laboratories in all the submitted AMR profiles of this strain. The most frequent antimicrobial missed by participants for this strain was ceftazidime-avibactam (n=22), followed by sulfamethoxazole (n=8), piperacillin-tazobactam (n=7) and amoxicillin-clavulanic acid (n=6). Other expected antimicrobials aztreonam and amikacin were missing in five and four AMR profiles each. There were 25 other cases of missing expected antimicrobials for this strain. In total, the number of missing antimicrobials throughout all submitted AMR profiles was 73. The expected but non-mandatory antimicrobial colistin was missing in 25 AMR profiles. Overall, 14 AMR profiles contained all expected antimicrobials without any unexpected antimicrobial. In total, 55 unexpected antimicrobials were reported for this strain, including ampicillin to which *K. pneumoniae* is intrinsically resistant. Ampicillin is also the most frequently reported antimicrobial for this strain (n=26), followed by fosfomycin (n=23) and tigecycline (n=6). The complete description of the results submitted by participants is provided in Table 27.

Table 27. Results of the *in silico* prediction of AMR profiles for each participant and for each type of dataset (BACT, DNA or SEQ), for strain EURGen-2024-04 (*K. pneumoniae*)

Laboratories	Sample type	Expected: Amikacin	Amoxicillin-clavulanic acid	Aztreonam	Cefepime	Cefotaxime	Ceftazidime	Ceftazidime-avibactam	Ciprofloxacin	Ertapenem	Gentamicin	Imipenem	Meropenem	Piperacillin-tazobactam	Sulfamethoxazole	Tobramycin	Trimethoprim	Colistin[a]	Unexpected: Ampicillin[d]	Fosfomycin	Tigecycline	Correct (nr.)	Missing (nr.)	Unexpected (nr.)	Expected non-mandatory (nr.)
EURGen-RLC-001	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-002	BACT	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	15	1	3	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16	0	3	1
EURGen-RLC-003[b]	SEQ	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-		x		15	1	1	0
EURGen-RLC-004[c]	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-				16	0	0	0
EURGen-RLC-005[b]	SEQ	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-				16	0	0	0
EURGen-RLC-009	BACT	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x	x		15	1	2	0
	DNA	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x	x		15	1	2	0
EURGen-RLC-010	BACT	x	x	x	x	x	x	-	x	x	-	x	x	x	x	x	-	-	x			13	3	1	0
	DNA	x	x	x	x	x	x	-	x	x	-	x	x	-	-	x	x	-	x			12	4	1	0
EURGen-RLC-011	BACT	x	x	x	x	x	x	-	x	x	x	x	x	x	-	x	x	-	x			14	2	1	0
	DNA	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x			15	1	1	0
EURGen-RLC-014	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		16	0	2	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		16	0	2	1
EURGen-RLC-016	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-018[c]	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-019	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	x	x		16	0	2	0
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	x	x		16	0	2	0
EURGen-RLC-020	BACT	x	-	x	x	x	x	-	x	x	x	x	x	-	-	x	x	x	x	x	x	12	4	3	1
	DNA	x	-	x	x	x	x	-	x	x	x	x	x	-	-	x	x	x	x	x	x	12	4	3	1
EURGen-RLC-021	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	16	0	2	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			16	0	1	1
EURGen-RLC-023	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-024[b]	SEQ	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-				16	0	0	0
EURGen-RLC-025	BACT	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-		x		15	1	1	0
	DNA	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-		x		15	1	1	0
EURGen-RLC-026	BACT	-	x	x	x	x	x	-	x	x	x	x	x	x	x	-	x	-	x	x		13	3	2	0
	DNA	-	x	x	x	x	x	-	x	x	x	x	x	x	x	-	x	-	x	x		13	3	2	0
EURGen-RLC-027	BACT	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	15	1	2	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-028[b]	SEQ	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x			15	1	1	0
EURGen-RLC-029	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
	DNA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-030[c]	BACT	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		15	1	2	1
EURGen-RLC-031	BACT	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x	x		15	1	2	0
	DNA	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x	x		15	1	2	0
EURGen-RLC-032[c]	BACT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				16	0	0	1
EURGen-RLC-033	BACT	x	-	x	x	x	x	-	x	x	x	x	x	-	-	x	x	-	x	x		12	4	2	0
	DNA	x	-	x	x	x	x	-	x	x	x	x	x	-	-	x	x	-	x	x		12	4	2	0
EURGen-RLC-034	BACT	-	-	-	-	-	-	-	-	-	-	x	x	-	-	-	-	-				2	14	0	0
	DNA	-	-	-	-	-	-	-	-	-	-	x	x	-	-	-	-	-				2	14	0	0
EURGen-RLC-036	BACT	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x	x		15	1	2	0
	DNA	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	-	x	x		15	1	2	0
EURGen-RLC-012[c]	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-015	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-017	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
	DNA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
EURGen-RLC-022[c]	BACT	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	NA	NA	NA	NA
Correct (nr.)		42	40	41	44	44	44	24	44	44	42	46	46	39	38	42	43	21	NA	NA	NA	**Total**			
Missing or UN (nr.)		4	6	5	2	2	2	22	2	2	4	0	0	7	8	4	3	25	26	23	6	663	73	55	21

NA: Not applicable; UN: Unexpected
Cells shaded in green (x): AMR profile reported for the antimicrobial
Cells shaded in red (-): AMR profile missing for the antimicrobial
Cells shaded in orange (x): AMR profile reported for the unexpected antimicrobial
Cells shaded in grey (/): participant did not submit AMR profile
[b] The laboratories that analysed only the sequencing data provided by EQA organizer
[c] The laboratories only analysed either BACT or DNA samples, and not both
[d] Intrinsic resistance (based on EUCAST Expected Phenotypes Version 1.2, January 2023)

The major discrepancies observed between expected and submitted results for the *in silico* prediction of AMR profiles were the lack of reporting predicted resistance towards aztreonam (n=50) in strains EURGen-2024-01 (*E. coli*), EURGen-2024-02 (*P.* aeruginosa) and EURGen-2024-04 (*K. pneumoniae*), ciprofloxacin (n=43) in all four strains and ceftazidime-avibactam (n=44) in strains EURGen-2024-01 and EURGen-2024-04.

Many laboratories did not report the expected antimicrobial aztreonam for strain EURGen-2024-02 (*P. aeruginosa*) (n=27). It was included in the expected results for this strain due to the presence of an extended-spectrum β-lactamase gene *bla*$_{\text{PME-1}}$, also reported by many laboratories as an expected AMR gene. However, the resistance towards aztreonam is missing from the output associated with *bla*$_{\text{PME-1}}$ in all three tools and associated databases (ResFinder, AMRFinderPlus and CARD-RGI). While preparing the expected results, aztreonam was also added to the expected AMR profile of the strain EURGen-2024-01 (*E. coli*), since the *bla*$_{\text{CTX-M}}$ gene was present in the strain. For self-evaluation it is important to note that there is conflicting evidence for CTX-M enzymes hydrolyzing monobactams. These discrepancies illustrate the need for laboratories to become familiar with underlying genetic mechanisms of resistance so that they can critically evaluate results from bioinformatics analyses and avoid "false negatives". This finding is furthermore supported by the absence of ceftazidime-avibactam from several results. That antimicrobial combination is present in the most used tool for prediction of AMR profiles, which was ResFinder. However, in the database of that tool, resistance towards ceftazidime-avibactam is not part of the output associated with the carbapenemase genes *bla*$_{\text{NDM-1}}$, *bla*$_{\text{NDM-5}}$, *bla*$_{\text{OXA-23}}$, and *bla*$_{\text{IMP-62}}$, which were part of the expected results. This error in the ResFinder database increased the difficulty of this prediction, especially because the antimicrobial exists in the database associated with other genes.

In case of missing expected antimicrobial ciprofloxacin in EURGen-2024-03 (*A. baumannii*) the problem arises due to the lack of database of PMs conferring AMR for this species in the ResFinder tool. In *A. baumannii*, mutation-based ciprofloxacin resistance tends to emerge primarily due to the PM *gyrA* S81L, followed by secondary mutations in the *parC* S84L, which were part of the expected results for the *A. baumannii* strain and were detected only by using AMRFinderPlus and CARD-RGI. Hence, the laboratories that only used ResFinder for detecting genetic AMR determinants will not be able to detect these PMs, consequently failing to predict the resistance to ciprofloxacin in this strain. For strain EURGen-2024-02 (*P. aeruginosa*), missing the expected antimicrobial tobramycin (n=19) was because many of these laboratories failed to detect *aac(6')-Ib* (or *aac(6')-Ib-Hangzhou* or *aac(6')-Ib3* or *aac(6')-Ib4* or *aac(6')-Ib9*).

In addition to understanding the genetic and molecular mechanisms of resistance, these problems support the need for laboratories to supplement their analysis with other bioinformatics tools and/or literature research, at least during the initial stages of implementation of WGS-based data analysis in their settings. Naturally, laboratories should also be familiar with the databases themselves to know if certain gene families or antimicrobial agents are not at all present. Moreover, these issues highlight the importance of participating in international genomic EQAs, since analysis of data from these exercises reveals these specific problems and allows or the benchmarking of the different bioinformatics pipelines used in different settings.

The problem of reporting unexpected antimicrobials was less prevalent during the 2024 EQA compared to the 2023 EQA. In the current EQA, six laboratories did not report any unexpected antimicrobial in all the strains for which they submitted results, while all laboratories reported unexpected antimicrobials in the previous EQA. Overall, 27 unexpected antimicrobials were reported by the participants for all four strains. Most of these problems were observed in the *A. baumannii* and *P. aeruginosa* strains and were mainly due to the reporting of antimicrobials for which the species are intrinsically resistant. In *A. baumannii* and *P. aeruginosa* strains, the β-lactams ampicillin, amoxicillin-clavulanic acid, cefotaxime and ertapenem were the most reported unexpected antimicrobials. Although these strains are resistant to these antimicrobials, resistance is due to the combination of intrinsic mechanisms such as the presence of chromosomal cephalosporinases and carbapenemases (*bla*$_{ADC}$, *bla*$_{CARB-2}$, and *bla*$_{OXA-51}$-type), and low permeability to certain antimicrobials (ertapenem). These antimicrobials should not have been reported for *A. baumannii* and *P. aeruginosa*, following the guidelines in the 2024 EQA protocol regarding the reporting of intrinsic resistance mechanisms. Similarly, the incorrect reporting of resistance towards fosfomycin, as discussed previously in relation to the detection of genetic determinants of AMR, are direct consequences of detection of intrinsic *fosA* gene, not proven to be associated with decreased susceptibility towards fosfomycin comparing to the wild-type phenotype in these species. Therefore, neither the genetic determinants nor the AMR profiles should be part of submitted results.

Finally, the only antimicrobial that was expected but non-mandatory to report was colistin in strain EURGen-2024-04 (*K. pneumoniae*). In this strain, colistin resistance was mediated by the chromosomal PM *mgrB W20R* but was only detected in AMRFinderPlus. Twelve laboratories predicted the colistin resistance in this strain and most of these laboratories used AMRFinderPlus for the detection of AMR determinants. This PM (and thus, the respective associated AMR profile) are not part of the ResFinder database, again defending the approach of using confirmatory bioinformatics tools and the need to become familiar with the genetic mechanisms mediating AMR in different species, and their respective presence or absence in the chosen bioinformatics tools. The participants should be familiar with the advantages and shortcomings of each bioinformatics tool and use more than one bioinformatics tool and database to confirm the presence of relevant genetic determinants.

4. FEEDBACK FROM PARTICIPANTS

The feedback survey was shared with the participants on 16th September 2024 and the deadline to submit the feedback was 30th September 2024. By the deadline, 12 laboratories completed the feedback survey and submitted their comments.

Regarding the usefulness of the 2024 EQA, the average score was 9 out of 10. Ten participants answered that the preliminary individual EQA evaluation reports they received in September were clear and useful, while two participants answered that the reports were not clear and useful. Nine participants answered that the individual quality assessment report of the sequences was clear and useful, while one participant answered that the report was not clear and useful, and two participants did not submit sequences for quality evaluation. Six participants answered that they took corrective actions based on the recommendations of the report while the other six participants answered that they did not take any action.

Respondents were able to include free text answers regarding any suggestions to make upcoming EQAs more useful. The responses received during the feedback survey, via email, and during physical network meeting in September 2024 are summarized in the following main points:

- One participant highlighted that *bla*$_{\text{CARB-2}}$ is not intrinsic in *P. aeruginosa* and provided literature evidence. Upon further investigation and literature review, the EQA providers agree that it is an acquired genetic determinant and should be reported if detected in *P. aeruginosa*.

- One participant suggested including phenotypic antimicrobial susceptibility testing (AST) as part of the EQA. The EURGen-RefLabCap EQAs focus exclusively on genomic analyses and therefore provision and evaluation of AST results is outside of the scope of these EQAs. However, participants are welcome to perform phenotypic AST of the strains shared within the EURGen-RefLabCap EQAs at their own discretion.

- One participant suggested developing schemes for each bacterial species included in the EQA listing the relevant antimicrobials and the important resistance mechanisms (genes, mutations, insertion, porin, etc.). While such a resource would be pertinent for educational purposes, the evaluation of knowledge regarding relevant antimicrobials is implied in the EURGen-RefLabCap EQAs, even if not scored directly, and the evaluation of knowledge regarding mechanisms of resistance is one of the main focuses of the EQAs.

5. CONCLUSION AND RECOMMENDATIONS

The results from the EURGen-RefLabCap 2024 EQA show that, throughout Europe, there is still a lack of uniformity regarding analysis of WGS data for public health purposes such as surveillance of important healthcare-associated pathogens. However, it is encouraging to see that many of the laboratories have followed some of the recommendations from the previous EQAs in EURGen-RefLabCap project such as incorporating more than one bioinformatics tool and/or database for the WGS analyses.

Some of the discrepancies observed between expected and reported results appeared to be due to variations between the type of bioinformatics tools and databases used, and to the lack of databases for the detection of plasmids and genetic AMR determinants for *Acinetobacter* and *Pseudomonas* species. However, these discrepancies should not be interpreted as a lack of knowledge and bioinformatics capacity by the participants, but instead underscore the need for international harmonization of bioinformatics approaches. Most of the responsibility towards harmonisation of bioinformatics tools and databases belongs to their curators, who should try to improve the databases and include PMs conferring AMR in *P. aeruginosa* and *A. baumannii* (and other species outside of the scope of the EURGen-RefLabCap project), they should strive for the synchronization of nomenclature and should engage in ongoing, active dialogue to ensure conformity between approaches.

In addition to the discrepancies caused by the differences in the bioinformatics tools and databases used, a significant number of the discrepancies between the expected and reported results were due to the laboratories not following the guidelines in 2024 EQA protocol for the reporting of results into the webtool. These might have been aggravated by insufficient knowledge about certain genetic mechanisms involved in AMR, leading to the reporting of unexpected AMR genes and chromosomal PMs, as well as the prediction of AMR towards unexpected antimicrobials. Additionally, certain expected antimicrobials were also not reported due to the insufficient knowledge about the underlying AMR mechanisms. This issue was mainly prevalent in the results reported for *A. baumannii* and *P. aeruginosa*, and to some extent, also for *K. pneumoniae*.

To increase local capacity for WGS-based analyses, the proposed actions are:

- Laboratories planning to implement or in the process of implementing WGS-based analysis in their settings should aim at using harmonized protocols such as the ones created during the EURGen-RefLabCap project[34]
- Laboratories currently using WGS could consider aligning their own protocols with other harmonized protocols, specifically by adhering to strict QC parameters and thresholds and by considering replacing some of their bioinformatics approaches
- Laboratories should ensure sufficient knowledge about the genetic mechanisms mediating AMR and other important genetic elements, especially the distinction between intrinsic and acquired ARGs, intrinsic phenotypic resistance in the relevant species, and the impact of each acquired ARG and chromosomal PM. In some cases, this will also rectify the discrepancies caused by the inaccuracy of bioinformatics databases (e.g., missing ceftazidime-avibactam from the AMR profile for carbapenemase genes in ResFinder database)
- Laboratories should analyse their results critically, for example by confirming the genomic location of the detected genes or plasmid replicons to confirm the presence of simultaneous variants or if this is an artifact of the bioinformatics tool

[34] https://www.eurgen-reflabcap.eu/protocols-and-guidelines

- Laboratories should be familiar with the bioinformatics tools and the contents of the associated databases they use, and one of the main objectives is to avoid false-negative results, such as assuming susceptibility towards antimicrobials because the database does not contain species-specific chromosomal PMs, or does not contain certain AMR genes, or does not contain all relevant antimicrobials in the predicted AMR profile
- Where possible, laboratories should always perform confirmatory testing by using additional bioinformatics tools to ensure that the information being reported is accurate and complete. Specifically, laboratories should use at least two tools and/or databases when identifying genetic AMR determinants and plasmid replicons. These tools should be curated and ideally have regular updates
- Laboratories should make sure that the results are accurately reported into electronic systems (or even paper-based systems) by ensuring a critical review during the submission process to avoid reporting errors such as typos
- Laboratories should communicate their suggestions, unusual and unexpected observations, and potential problems to the curators of the respective bioinformatics tools and databases.

Participants of the EURGen-RefLabCap 2024 EQA who did not obtain results in full agreement with expected results are invited to repeat the analyses with the bioinformatics approaches and thresholds used to generate the expected results (as described in the "Methods" section of this report). Participants are welcome to contact the EQA organizers for support in troubleshooting in case they do not obtain the full set of expected results upon re-analyses. Continued participation in genomic EQAs, the use of well-defined quality control parameters and respective thresholds, and the use of benchmarking datasets to validate different bioinformatics approaches are strategies that further contribute to the increase of local, national, and European capacity for WGS-based analysis and surveillance of important healthcare-associated pathogens.

Lessons learned from delivering the EURGen-RefLabCap EQAs will be carried over to the planning and delivery of relevant activities in the European Reference Laboratory for public health on AMR (EURL-PH-AMR) designated to a consortium led by SSI and composed of DTU and the Clinical Microbiology Laboratory, Region Kronoberg - EUCAST Development Laboratory, Sweden.

6. APPENDICES

6.1. Appendix 1: The quality control parameters included for the evaluation of Illumina sequences submitted by the participants in the 2024 EQA

Parameters	Description
Number of reads	The number of reads refers to the sequence yield, how much was sequenced.
Number of reads after trimming	The number of reads remaining after quality trimming and common adapter removal.
Q-score R1 / R2	Average quality score of the bases in the forward / reverse reads.
Number of reads mapped to the reference chromosome	The number of reads which map directly to the chromosome of the reference genome.
Number of reads mapped to plasmid N (if any)	The number of reads which map directly to each specified plasmid of the reference genome.
Number of reads mapped to the complete genome	The number of reads which map directly to the reference genome.
Proportion of reads mapped to the reference DNA sequence (%)	The proportion of reads which map directly to the reference genome. This cannot exceed 100%.
Coverage of the reference genome/chromosome/plasmid N (%)	The extent to which reads have covered the entirety of the reference genome/chromosome/plasmid N. This cannot exceed 100%.
Coverage 5/10/20x of genome/chromosome/plasmid N (%)	The coverage of minimum depth X of each genomic element. This cannot exceed 100%.
Depth of coverage: Complete genome / Chromosome / Plasmid N	Number of base pairs sequenced divided by the total size of the reference genome/chromosome/plasmid N. This number can be rounded to the nearest integer. In essence, this number describes the number of times the sequenced base pairs cover the reference DNA and is often ended with an "x" (e.g. 30x).
Average insert size	The average length of DNA between the adapters. (only calculated for paired end sequencing)
Size of assembled genome	The total size of all contigs in base pairs.
Size of assembled genome (200 bp)	The total size of all contigs in base pairs, only counting contigs longer than 200 bp
Size of assembled genome per total size of DNA sequence (%)	Size of assembly compared to the size of the reference genome. Should be as close to 100 % as possible.
Size of assembled genome per total size of DNA sequence (contigs above 200 bp) (%)	Size of assembly compared to the size of the reference genome, only counting contigs more than 200bp. Should be as close to 100% as possible.
Total number of contigs	The total number of contigs assembled.
Number of contigs > 200 bp	The total number of contigs assembled which have a sequence length longer than 200 bp.
N50	The N50 is defined as the length of the contig, for which the sum of all contigs of that length or longer equals at least 50% of the sum of all contigs.

Parameters	Description
NG50	The NG50 is defined as the length of the contig, for which the sum of all contigs of that length or longer equals at least 50% of the reference genome size.
Proportion of cgMLST match/not found/imperfect hit/wrong allele	Estimate of alleles that can be correctly called from the produced assembly of the sequence data. Expected alleles predicted from the reference genome are compared with the best hit from the assembly.

6.2. Appendix 2: The quality control parameters included for the evaluation of ONT sequences submitted by the participants in the 2024 EQA

Parameters	Description
Number of (filtered) reads	The number of reads describes the sequence yield, how much was sequenced. The filtered number refers to after filtering to a minimal length of 500bp and average quality score of 12.
Number of (filtered) bases	The total number of base pairs in your reads. The filtered number refers to after filtering to a minimal length of 500bp and average quality score of 12.
Longest read	Length in base pairs of longest read.
Shortest read	Length in base pairs of shortest read.
N50 (filtered) read length	The N50 of all reads after filtering. The N50 is defined as the length of the read, for which the sum of all reads of that length or longer equals at least 50 % of the sum of all base pairs.
Mean/Median (filtered) read length	Mean/median length of all reads, before and after filtering.
Mean/Median (filtered) read quality	Mean/median Q-score of all reads, before and after filtering.
(Filtered) reads >500bp	Number of reads larger than 500bp before and after filtering. Number above thresholds of 1000, 2000, 5000 and 10000bp is likewise stated.
(Filtered) reads quality >10	Number of reads with an average Q-score above 10, before and after filtering. Likewise stated for thresholds of 12 and 20. Note, filtering removes reads of average Q-score <12.
Number of mapped reads	Total number of reads mapped to the reference genome.
Mapped to chromosome/plasmid N	Number of reads mapping to the specific genomic component.
Total assembly size	Total number of base pairs in the assembly.
Number of contigs	The number of produced contigs compared to the number expected in the reference (chromosome + number of plasmids), shown as a fraction.
Number circularized	Number of contigs reported to be circularized by the assembler.
MLST	Identified MLST
Coverage of the reference genome/chromosome/plasmid N (%)	Proportion of the reference genome, chromosome or plasmid (N) covered by reads (this cannot exceed 100%)
Coverage 20/30/40/50x of the reference genome/chromosome/plasmid N (%)	Proportion of the reference genome, chromosome or plasmid N, covered by at least X times of reads. (This cannot exceed 100%).

6.3. Appendix 3: Methods reported by the participants for prediction of MLST in the 2024 EQA

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-001	web-based	MLST-2.0.9 (2022-05-11)	(2023-06-19)	default	https://cge.food.dtu.dk/services/MLST/
EURGen-RLC-002	local	MLST version 2.23.0	-	-	-
EURGen-RLC-003	-	-	-	-	-
EURGen-RLC-004	web-based	PubMLST	PubMLST	default	https://pubmlst.org/
EURGen-RLC-005	-	-	-	-	-
EURGen-RLC-009	Web-based pipeline	CGE MLST 2.0 Software version: 2.0.9 (2022-05-11)	Database version: (2023-06-19)	default	https://cge.food.dtu.dk/services/MLST/
EURGen-RLC-010	Local	MLST 2.23.0 (https://github.com/tseemann/mlst)	CGE mlst_db (version: 2023-06-19)	default	Github: https://github.com/tseemann/mlst ¤ CGE https://bitbucket.org/genomicepidemiology/mlst_db/src/master/
EURGen-RLC-011	local	srst2	publicly available	default parameters	https://github.com/katholt/srst2
EURGen-RLC-012	-	-	-	-	-
EURGen-RLC-014	local	Ridom SeqSphere+ 10.0	-	default parameters	-
EURGen-RLC-015	local	tseemann / mlst via *AMR	-	-	https://github.com/tseemann/mlst
EURGen-RLC-016	local	SeqSphere+ (Ridom), v9.0.10	SeqSphere+ (Ridom), v9.0.10	-	https://www.ridom.de/seqsphere/
EURGen-RLC-017	Local, pipeline on SeqSphere	SeqSphere 10.0.0	SeqSphere 10.0.0 E.coli MLST Warwick, A.baumanii MLST Oxford	default	-
EURGen-RLC-018	web-based pipeline	MLST 2.0, Software version: 2.0.9 (2022-05-11) ¤	PubMLST.org. Database version: (2023-06-19)	default parameters ¤	https://cge.food.dtu.dk/services/MLST/
EURGen-RLC-019	local	mlst,2.19.0	mlst,2.19.0	default	https://github.com/tseemann/mlst
EURGen-RLC-020	-	Ridom Seqsphere, version 10.0.0	-	Default	https://www.ridom.de/seqsphere
EURGen-RLC-021	Local Seqsphere based pipelines	Seqsphere version 10.0.0 (2024-04)	Seqsphere version 10.0.0 (2024-04)	default	-
EURGen-RLC-022	-	-	-	-	-

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-023	local	mlst v.2.23.0	publicly available mlst v.2.23.0	default	https://github.com/tseemann/mlst
EURGen-RLC-024	-	-	-	-	
EURGen-RLC-025	local	mlst v.2.23.0 (INNUca v.4.2.3 Pipeline)	PubMLST database updated on 24/06/2024	default parameters	https://github.com/tseemann/mlst; https://github.com/B-UMMI/INNUca
EURGen-RLC-026	web-based	publicly available software	MLST 2.0 Software version: 2.0.9	Default	https://cge.food.dtu.dk/services/MLST/
EURGen-RLC-027	Local and Web-based	Ridom SeqSphere+	-	-	-
EURGen-RLC-028	-	-	-	-	-
EURGen-RLC-029	local	SeqSphere v10.0.2	PubMLST via SeqSphere+ (Ridom)	default	https://pubmlst.org/, https://www.ridom.de/seqsphere/
EURGen-RLC-030	Local pipeline	mlst, 2.22.1	PubMLST, BIGSdb Version 1.46.0	Default parameters	https://github.com/tseemann/mlst ¤ https://pubmlst.org
EURGen-RLC-031	web-based	Galaxy, staramr tool and CGE web	Galaxy, staramr tool	Default parameters	https://usegalaxy.eu/
EURGen-RLC-032	-	-	-	-	-
EURGen-RLC-033	mlst-cge 2.0.9	mlst-cge 2.0.9	mlst-cge 2.0.9	default	https://bitbucket.org/genomicepidemiology/mlst
EURGen-RLC-034	local	Ridom Seqsphere plus version 9.08 EULA	Warwick for E.coli and Pseudomonas aeruginosa, Oxford for Acinetobacter baumannii and Pasteur for Klebsiella pneumoniae	default except for Acinetobacter baumannii: selected setting: Force using best match if multiple matches found within the treshold	NA
EURGen-RLC-036	local	mlst (tseemann) v2.23.0	pubmlst 2024-03-27	default	https://github.com/tseemann/mlst

-; Information was not provided by the laboratory

6.4. Appendix 4: Methods reported by the participants for detection of plasmid replicons in the 2024 EQA

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-001	web-based	PlasmidFinder-2.0.1 (2020-07-01)	(2023-01-18)	default	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-002	local	PlasmidFinder with Abricate 1.0.1; Plassembler 1.6.2	-	-	-
EURGen-RLC-003	-	-	-	-	-
EURGen-RLC-004	local	PlasmidFinder v2.0.1 (2020-07-01)	PlasmidFinder database (2023-01-18)	90% identity, 80% coverage	https://bitbucket.org/genomicepidemiology/plasmidfinder/src/master/
EURGen-RLC-005	-	-	-	-	-
EURGen-RLC-009	web-based pipeline	CGE PlasmidFinder 2.1 Software version: 2.0.1 (2020-07-01)	Database version: (2023-01-18). Database for Enterobacteriales	minimum identity >80% to identify col-plasmids, according to paper of Carattoli et al.	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-010	CGE Plasmidfinder web based	CGE PlasmidFinder 2.1 (Software version: 2.0.1)	Database version: plasmidfinder_db 2.1.0, https://bitbucket.org/genomicepidemiology/plasmidfinder_db/src/master/	Minimum identity 95% and miinimum coverage 60%	https://bitbucket.org/genomicepidemiology/plasmidfinder.git/src
EURGen-RLC-011	web-based	CGE plasmid finder	CGE plasmid database	default	https://www.genomicepidemiology.org/
EURGen-RLC-012	-	-	-	-	-
EURGen-RLC-014	local	Ridom SeqSphere+ 10.0	-	default parameters	-
EURGen-RLC-015	local	PlasmidFinder via *AMR	PlasmidFinder db	default	-
EURGen-RLC-016	Local	plasmidfinder, version 2.1.0	plasmidfinder database 2.1.0	default	https://bitbucket.org/genomicepidemiology/plasmidfinder/src/master/
EURGen-RLC-017	Local, pipeline on SeqSphere	MOB-recon version 3.1.4	MOB-recon version 3.1.4	default	-
EURGen-RLC-018	web-based pipeline	PlasmidFinder 2.1, Software version: 2.0.1 (2020-07- ¤ 01)	Database version: (2023-01-18)	default parameters	https://cge.food.dtu.dk/services/PlasmidFinder/

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-019	local	plasmidfinder,2024-06-18; mob-suite,3.0.3	plasmidfinder,2024-06-18; mob-suite,3.0.3	default	https://github.com/phac-nml/mob-suite, https://bitbucket.org/genomicepidemiology/plasmidfinder/src/master/
EURGen-RLC-020	-	Ridom Seqsphere, version 10.0.0	-	Default	-
EURGen-RLC-021	web-based	PlasmidFinder 2.0.1 (CGE tool)	PlasmidFinder 2.0.1 (CGE tool)	default	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-022	-	-	-	-	-
EURGen-RLC-023	local	PlasmidFinder v.2.1.6	Publicly available Plasmidfinder v.2.1.6 ¤	minimum coverage 90% and minimum identity 90%	https://bitbucket.org/genomicepidemiology/plasmidfinde ¤ r/src/master/
EURGen-RLC-024	-	-	-	-	-
EURGen-RLC-025	local	ABRicate v.1.0.1	Plasmidfinder database updated on 24/06/2024	default parameters	https://github.com/tseemann/abricate
EURGen-RLC-026	web-based pipeline	publicly available software	PlasmidFinder 2.1 Software version: 2.0.1	Default	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-027	Web-based pipeline	PlasmidFinder 2.0.1 (2020-07-01)	Database version: (2023-01-18)	default (60% length, 95% identity)	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-028	-	-	-	-	-
EURGen-RLC-029	web-based	PlasmidFinder v2.0.1	Database version: (2023-01-18)	default	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-030	Local pipeline	PlasmidFinder, 2.0.1	plasmidfinder_db, version 2023-01-18	90% minimum identity.	https://bitbucket.org/genomicepidemiology/plasmidfinder_db/src/master
EURGen-RLC-031	web-based	Galaxy, staramr tool (PlasmidFinder)	PlasmidFinder	Default parameters	https://usegalaxy.eu/
EURGen-RLC-032	-	-	-	-	-
EURGen-RLC-033	web-based	PlasmidFinder 2.1	Enterobacteriales	default	https://cge.food.dtu.dk/services/PlasmidFinder/
EURGen-RLC-034	local	PlasmidFinder 2020-02-07	Plasmidfinder database version: 2.1.0	minimum length 60% and minimum identity 95%	https://bitbucket.org/genomicepidemiology/workspace/projects/DB
EURGen-RLC-036	local	mob_suite v3.1.8	ncbi_plasmid_db v 2024-02-08	default	https://github.com/phac-nml/mob-suite

-; Information was not provided by the laboratory

6.5. Appendix 5: Methods reported by the participants for detection of genetic determinants of AMR and prediction of AMR profiles in the 2024 EQA

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-001	web-based and local	ResFinder-4.5.0 (2024-03-22); AMRFinderPlus-3.11.2	ResFinder: (2024-03-22); AMRFinderPlus: 2022-12-19.1	default	http://genepi.food.dtu.dk/resfinder
EURGen-RLC-002	local	Resfinder, NCBI AMRFinder, CARD with Abricate 1.0.1	-	-	-
EURGen-RLC-003	-	-	-	-	-
EURGen-RLC-004	local and web-based	abritamr v1.0.18 and ResFinder 4.5.0	amrfinderplus database 2024-05-02 ¤ ResFinder database: (2024-03-22) ¤ PointFinder database: (2024-03-08)	default	https://github.com/MDU-PHL/abritamr ¤ http://genepi.food.dtu.dk/resfinder
EURGen-RLC-005	-	-	-	-	-
EURGen-RLC-009	web-based pipeline	ResFinder 4.5.0 ResFinder software: (2024-03-22)	ResFinder database: (2024-03-22)	minimum identity 95%	http://genepi.food.dtu.dk/resfinder
EURGen-RLC-010	Local	ResFinder (verison: 4.5.0), PointFinder and AMRFinderPlus (verison: 3.11.18)	Resfinder_db version: 2.3.2 ¤ Poinfinder_db version: 4.1.0 ¤ AMRFinderPlus_db version: 15.1	default	https://git@bitbucket.org/genomicepidemiology/resfinder_db.git db_resfinder, git clone https://git@bitbucket.org/genomicepidemiology/pointfinder_db.git ¤ https://github.com/ncbi/amr
EURGen-RLC-011	both	srst2 and CGE	CARD, ARGAnnot and ResFinder	default	-
EURGen-RLC-012	-	-	-	-	-
EURGen-RLC-014	local	Ridom SeqSphere+ 10.0	-	default parameters	-
EURGen-RLC-015	web-based	ResFinder	-	default	http://genepi.food.dtu.dk/resfinder

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-016	local	AMRFinderPlus, version 3.12.8 ¤ ResFinder, version 4.4.2	AMRFinderPlus database 2024-01-31.1 ¤ ResFinder database 2.3.2	Default for both	AMRFinderPlus: https://github.com/ncbi/amr ¤ AMRFinderPlus database: https://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/AMRFinderPlus/database/latest/ ¤ ResFinder: https://bitbucket.org/genomicepidemiology/resfinder/src/master/ ¤ ResFinder database: https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/
EURGen-RLC-017	Local, pipeline on SeqSphere	AMRFinderPlus software version: 3.11.26	AMRFinderPlus database version: 2023-11-15.1	default parameters for detection, We only reported >99% aligned overlap and >99% identification as this is what we do for our clinical isolates.	-
EURGen-RLC-018	web-based pipeline	ResFinder 4.5, AMRFinderPlus ¤ NCBI Antimicrobial Resistance Gene Finder ¤ (Galaxy Version 3.11.26+galaxy1) ¤	¤ ResFinder database: (2024-03-22), AMRFinderPlus ¤ NCBI Antimicrobial Resistance Gene Finder ¤ (Galaxy Version 3.11-2022-12-19.1) ¤	default parameters	http://genepi.food.dtu.dk/resfinder, https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fiuc%2Famrfinderplus%2Famrfinderplus%2F3.12.8%2Bgalaxy0&version=latest
EURGen-RLC-019	local	AMRFinderPlus v3.12.8, ¤ ResFinder v4.5.0 (database version 2024-06-18), and RGI v6.0.3	AMRFinderPlus v3.12.8, ¤ ResFinder v4.5.0 (database version 2024-06-18), and RGI v6.0.3	default	AMRFinderPlus v3.12.8, ¤ ResFinder v4.5.0 (database version 2024-06-18), and RGI v6.0.3
EURGen-RLC-020	-	Ridom Seqsphere, version 10.0.0	-	Default	-
EURGen-RLC-021	both	AMRFinderPlus (3.11.26); Resfinder (4.5.0; 2024-03-22)	AMRFinderPlus (2023-11-15.1); Resfinder (4.5.0; 2024-03-22)	default	http://genepi.food.dtu.dk/resfinder
EURGen-RLC-022	-	-	-	-	-

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-023	local	AMRFinderPlus v.3.12.8, Kleborate v.2.3.2	AMRFinderPlus database version 2024-01-31.1	minimum coverage 90% and minimum identity 90%, ¤ secondarily minimum coverage 60% and minimum ¤ identity 60%	https://github.com/ncbi/amr ¤ https://github.com/klebgenomics/Kleborate/wiki
EURGen-RLC-024	-	-	-	-	-
EURGen-RLC-025	local	ABRicate v.1.0.1 and AMRFinderPlus 3.12.8	ABRicate v.1.0.1: ResfInder and NCBI databases updated on 24/06/2024; AMRFinderPlus 3.12.8 database v.2024-05-02.2	default parameters	https://github.com/tseemann/abricate; https://github.com/ncbi/amr
EURGen-RLC-026	web-based pipeline	publicly available software	ResFinder 4.5.0	Default	https://genepi.food.dtu.dk/resfinder
EURGen-RLC-027	Local and Web-based	Ridom SeqSphere+	-	default (60% length, 90% identity)	http://genepi.food.dtu.dk/resfinder
EURGen-RLC-028	-	-	-	-	-
EURGen-RLC-029	both	AMRFinder, v3.12.8 ¤ ResFinder, v4.5.0	NCBI AMRFinder DB v(2024-05-02.2) ¤ ResFinder DB v(2024-03-22)	default	http://genepi.food.dtu.dk/resfinder ¤ https://github.com/theiagen/public_health_bioinformatics/issues/334
EURGen-RLC-030	Local pipeline	AMRFinderPlus, ResFinder	AMRFinder database (2024-05-02) ¤ ResFinder database v2.3.2 (2024-04-25)	Default parameters	https://bitbucket.org/genomicepidemiology/resfinder_db, ¤ https://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/AMRFinderPlus/database/latest, ¤ https://bitbucket.org/genomicepidemiology/resfinder/src/master/, ¤ https://github.com/ncbi/amr ¤
EURGen-RLC-031	web-based	Galaxy, staramr tool (ResFinder) and CGE web	ResFinfer	Default parameters	https://usegalaxy.eu/
EURGen-RLC-032	-	-	-	-	-

Laboratory	Pipeline type	Software	Database	Parameters of the software	URL of the software or database
EURGen-RLC-033	local	abritamr	AMRFinder Plus	default	https://github.com/MDU-PHL/abritamr
EURGen-RLC-034	local	Resfinder 4.4.2	Resfinder database versie : 2.2.1	minimum length 60% and minimum identity 95%	https://bitbucket.org/genomicepidemiology/workspace/projects/DB
EURGen-RLC-036	local	ncbi-amrfinderplus v3.12.8	ncbi-amrfinderplus v2024-01-31	default	https://github.com/ncbi/amr

-; Information was not provided by the laboratory

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