

AMR Rules: Interpretive Standards for AMR Genotypes

ESGEM-AMR Working Group Technical Guidance for Defining Interpretive Rule Sets

Background, rationale and workplan for the ESGEM-AMR working group are described [here](#).

Members are encouraged to use their own knowledge and expertise to propose rule sets for their assigned species, as long as they can provide sufficient evidence to justify the rules.

This document lays out technical details regarding the proposed data standard for the rule sets, guidance on technical issues and standards of evidence needed to add rules, and a suggested protocol for populating rule sets.

NOTE: the notation R^{NWT} , R^{WT} is used in these descriptive documents for clarity, to avoid spaces, and in line with the EUCAST committee nomenclature. However as the interpretive rules need to be encoded in simple text they cannot include superscript, therefore they are written as 'nwt R', 'wt R', etc instead. The two notations have identical meaning, i.e. ' R^{NWT} ' should be read interchangeably with 'nwt R' as meaning 'wildtype resistant'.

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Objectives

The interpretive standards aim to capture all exceptions to the generalized interpretation of ‘presence of gene X’ implies ‘R^{NWT} or I^{NWT} to drug Y’. Ultimately, they should also differentiate R^{NWT} from I^{NWT}, and interpret combinations of genes. However, the initial priority will be creating rule sets that clearly delineate **core genes** associated with ‘**wildtype**’ **phenotypes** for each species (see Table 1 below for basic format).

Initial development will work species-by-species, generating **one rule set for each species**, which can be combined together into a single resource, via the [AMRules](#) project.

FAIR and Open Principles

The overall project adheres to [FAIR](#) principles to ensure the interpretive standards developed are findable, interoperable, accessible, and reusable.

- Rule sets will be made **publicly available** via open-access repositories under a permissive license (GNU General Public License v3.0).
- They will be **versioned** via numbered releases, and issued with stable document object identifiers (DOIs).

Rule sets and associated tools should be **interoperable with existing resources** for AMR genotype analysis as far as possible, including use of common sequence identifiers, standard gene nomenclature, and data formats.

- The primary reference database for nomenclature and sequence accessions of AMR determinants will be NCBI’s Reference Gene Catalog ([refgene](#)) and corresponding Reference Gene [Hierarchy](#).
- The primary reference database for nomenclature for drugs and drug classes will be the Comprehensive Antibiotic Resistance Database (CARD) [Antibiotic Resistance Ontology](#).
- Preliminary [code](#) has been developed for annotating the gene-level reports output by the [AMRFinderPlus](#) tool, which uses NCBI refgene and gene hierarchy for AMR determinants.
- Code will be developed to be interoperable with the [hAMRonization](#) format, to facilitate compatibility with the outputs of [CARD RGI](#), [ResFinder](#), and >12 other AMR genotyping tools whose outputs can be readily converted to [hAMRonization format](#). However in order for rule sets to be used with the outputs of tools that do not use NCBI refgene as the primary database, additional code/tools will be needed to harmonize sequence identifiers to those used to specify rule sets.

General Guidance and Priorities

Wildtype phenotypes

The initial focus of ESGEM-AMR will be creating rule sets that clearly delineate **core genes** associated with '**wildtype**' phenotypes for each species, where that phenotype is:

(1) Wildtype / intrinsic / expected resistant (R^{WT})

- This will usually be reflected in the EUCAST [Expected Resistance](#) and [Expert Rules](#) for the species. Note that where a species has Expected Resistance to a drug, there are no breakpoints or ECOFFS set for that species-drug combination as it is not clinically relevant, although sometimes [MIC distribution data](#) is recorded. Where it is not yet recognised that the wildtype phenotype is resistant, there may be an ECOFF and possibly a breakpoint with which to assess resistance from MIC data, and in some cases there may be a genus- or family-level breakpoint.
- Example: Wildtype alleles of core chromosomal gene *blaSHV* in *Klebsiella pneumoniae* hydrolyse penicillins, resulting in the [wildtype MIC distribution](#) for ampicillin exceeding the Enterobacterales R breakpoint of >8 mg/L. This is reflected in the EUCAST Expected Resistances (to ampicillin/amoxicillin and ticarcillin), Expert Rules (report as piperacillin resistant, regardless of test result), and lack of MIC distribution data for penicillin or ticarcillin for *Klebsiella pneumoniae*.

(2) Wildtype susceptible (S^{WT})

- This represents species-drug combinations for which the wildtype population has an MIC distribution that is below the S breakpoint, but that contains a core genetic marker that in another species produces an MIC distribution that is above the S or R breakpoint.
- Example: The *oqxAB* operon is a core locus conserved in *Klebsiella pneumoniae* chromosomes, where presence of wildtype alleles is not associated with clinical resistance to any antimicrobials. However when mobilised to other organisms such as *E. coli*, this locus is often hyper-expressed and associated with increased MIC or even clinical resistance to ciprofloxacin and other drugs. This is reflected in EUCAST Expected Resistances in the sense that these do **not** record ciprofloxacin as an expected resistance of *Klebsiella pneumoniae*. The [wildtype MIC distribution](#) for ciprofloxacin in *K. pneumoniae* mostly falls below the S breakpoints for ciprofloxacin in Enterobacterales (≤ 0.25).

Acquired phenotypes

Where relevant and supported by sufficient evidence, rules concerning the interpretation of **acquired determinants** may also be included. However in most cases such rules will need to be defined systematically, based on assessment of evidence for AMR determinants both **individually and in combination**. This will require high-volume high-quality genome-phenotype data, which is not yet available for most organisms and would need to be generated. This will be the focus of Phase 2 of the AMRrules initiative.

Format Specification

Rule sets will follow a **standard specification**, based on the one proposed [here](#) and developed in this [template](#), although technical details will be further developed and refined by the working group.

Each row defines a rule for interpreting the presence of one **gene** (or specific allele thereof) in one **species**, in terms of its expected genetic context (core or acquired) and its expected effect on **resistance category** for one **drug**.

Each row must have supporting **evidence** for the rule in the form of a PubMed identifier (**PMID**) for a peer-reviewed article, and an explanatory **note** summarizing the interpretation. Where a rule is made based on new unpublished data analysis, the supporting evidence should be submitted for review by the working group, and preprinted or published as soon as possible.

In principle there should be **one row per species/gene/drug combination**, for (i) clarity of interpreting and parsing the rules; and (ii) for clarity of recording evidence for each specific rule and its relevance to a given species, gene and drug. In reality there may be cases where a rule clearly applies across a higher taxonomic level, or across gene group, or across a drug class; guidance on specifying such rules are discussed below, and will be further developed during the initial development of rule sets.

For any given species, a rule should be included for **every** core gene matching a known resistance determinant (i.e. every core gene that appears in an AMRFinderPlus report), as the phenotypes of core genes should always be discernable from matched genome-phenotype data and/or an assumed link with Expected Resistance phenotypes defined by EUCAST, even in the absence of primary mechanistic literature on the topic.

In principle, a species' rule set should include a rule for every acquired gene in the refgene database where that gene has been reported in the species, but presence of that gene does not modify the phenotype for the associated drug/class in this organism. However it is anticipated that, for most species, there is not yet sufficient evidence to complete rule sets for all possible acquired genes; and acquired genes that lack functional evidence in this specific organism should not be included (see General Guidance above).

Required fields

Required fields are given in **Table 1**, populated with the exemplar rule set for *Klebsiella pneumoniae*; field definitions and guidance on completing them are given below.

A copy of this example, and the specification for each field, is also available in this spreadsheet [template](#).

Table 1. Proposed format for organism-specific rules, populated with example rules for *Klebsiella pneumoniae*.

organism	gene	context	drug	category	PMID	note
s__Klebsiella pneumoniae	blaSHV	core	penicillins	wt R	32284385	Specific alleles can be ESBL, these are mostly mobile
s__Klebsiella pneumoniae	oqxA	core	ciprofloxacin	wt S	30834112	Wildtype core gene, not expected to confer multiple drug resistance unless mobilised under strong promoter
s__Klebsiella pneumoniae	oqxB	core	ciprofloxacin	wt S	30834112	Wildtype core gene, not expected to confer multiple drug resistance unless mobilised under strong promoter
s__Klebsiella pneumoniae	fosA5_fam	core	fosfomycin	wt S	33128341	Wildtype core gene, not expected to confer multiple drug resistance unless mobilised under strong promoter (fosA5_fam is the parent node for K. pneumoniae chromosomal fosA)
s__Klebsiella pneumoniae	fosA5	core	fosfomycin	wt S	25441705	Wildtype core gene, not expected to confer multiple drug resistance unless mobilised under strong promoter
s__Klebsiella pneumoniae	fosA6	core	fosfomycin	wt S	27261267	Wildtype core gene, not expected to confer multiple drug resistance unless mobilised under strong promoter
s__Klebsiella pneumoniae	fosA10	core	fosfomycin	wt S	32431524	Wildtype core gene, not expected to confer multiple drug resistance unless mobilised under strong promoter

Organism

The organism to which the rule applies, which would normally be to the level of species.

The reference taxonomy for confirming species from a genome sequence will be the [Genome Taxonomy Database](#) (GTDB).

Note that GTDB taxonomic names use prefixes to indicate the taxonomic level to which a name belongs, e.g. the species *Klebsiella pneumoniae* has the prefix 's__' to indicate species, and is thus designated as "s__Klebsiella pneumoniae".

Future considerations:

- How to specify species complex? GTDB doesn't have internal nodes between the level of genus and species, and there is no other reference standard. The AMR R package has this set of [micro-organism groups](#) which is used to apply AST interpretation rules.
- Allow a list of species within the species cell? To allow a rule to be specified that applies to a subset or species within a genus. E.g. EUCAST Expected rules are sometimes specified for a set of two or more species. This could also provide a solution to the species complex question. However need to be mindful that this complicates the clear provision of evidence to support each rule and its taxonomic scope.

Gene

Gene symbol for a node in the [NCBI Reference Gene Hierarchy](#).

This has been selected over ‘allele’ or ‘gene family’ name as nomenclature standards are not consistent across AMR gene families; some entries lack ‘allele’ names; and node names clearly demarcate clusters of related variants with shared function for which it makes sense to specify a single rule. Most nodes in the gene hierarchy (~95%) have a one-to-one correspondence with a single ‘gene family’ name and/or ‘allele’ name in [refgene](#), which users will recognise as common names in the literature.

NCBI AMRFinderPlus uses the gene hierarchy but does not by default include the node name in the output file; we recommend users turn this on by setting the parameter `--print_node` when running the tool. However files generated without this switched on can trivially be annotated with node by using the sequence accession to look up the node name, for subsequent rule interpretation.

If a rule applies to all alleles of a given gene (e.g. all *blaSHV* alleles are expected to confer resistance to penicillins, although only some are ESBL), the rule should be specified using the corresponding node in the Reference Gene Hierarchy (e.g. node: ‘[blaSHV](#)’ is the parent for all numbered *blaSHV* alleles in refgene, hence this is used in the *Klebsiella pneumoniae* specification for wildtype resistance to penicillins in Table 1 above). If no suitable parent node exists, a rule can be specified using * as a wildcard, e.g. ‘*blaSHV**’.

Context

Indicates the genomic context of the gene within the specified organism, i.e. whether the gene is ‘core’ or ‘acquired’.

The working definition of ‘core’ gene is present (>90% identity, >90% length) in (i) the chromosome of >95% of genomes of this species (defined as above using GTDB); and (ii) the chromosome at >95% isolates that have wildtype AST profiles.

Future considerations:

- When assessing the frequency of a gene within a species, care must be taken with (i) taxonomic definitions (e.g. be aware of species vs species complex etc); and (ii) intraspecies diversity and representativeness of the available genome set.
- E.g. for *Acinetobacter baumannii*, genome databases are dominated by GC1 and GC2 clonal complexes which are highly resistant, such that genes which are core to those clones but lacking from other members of the species may have >95% frequency in the genome database. Rules will need to be developed around this, e.g. dereplicate to N<100 genomes per lineage (defined as sequence type or mash-distance cluster or similar), with at least N>20 unique lineages represented.

Drug

The name of the drug that the rule applies to.

The reference for drug names is the [Antibiotic Resistance Ontology](#) (part of the Comprehensive Antibiotic Resistance Database, [CARD](#)). The rule sets should prioritize rules for drugs of clinical relevance to treatment of the species and with EUCAST clinical breakpoints, rather than being exhaustive. Where rules apply to multiple drugs, they should be specified in separate rows, with individual references for each gene-drug combination.

For discussion: for beta-lactamases, rules may be specified for individual drugs (preferred; one drug per row, with evidence for each drug) or to a functional group ([monobactams](#), [penicillins](#), early (1st-2nd generation) cephalosporins, extended-spectrum (3rd-generation) cephalosporins, [carbapenems](#); one group per row, with evidence for each group). Note that resistance to beta-lactam drugs outside these groups still need to be specified individually (e.g. 4th generation cephalosporin [cefepime](#), 5th generation cephalosporin [ceftaroline](#), siderophore cephalosporin [cefiderocol](#), and all beta-lactamase plus inhibitor combinations).

Future considerations:

- Exceptions could be made to the one-rule-per-drug principle for beta-lactamases, which may be specified either as applying to individual drugs (preferred; one drug per row, with evidence for each drug) or to a functional group. However it is not obvious how best to define functional groups, as classification systems for beta-lactamase enzymes (e.g. Bush-Jacoby functional subgroups) do not map directly onto drug names.
- Recommend focusing on specifying rules for individual drugs but prioritizing those for which EUCAST specifies breakpoints and that are on the essential medicines list. For Enterobacterales, this would mean 23 drugs in 10 classes, see [summary](#). One might propose specifying rules for these 10 classes in future.
- The short-term solution proposed above is to allow the specification of rules for five groups of drugs which are clinically useful to distinguish and map broadly to different enzyme activities, meaning it will often be logical to specify a single rule that links a genetic determinant to the drug group. Resistance to drugs outside these groups (Cefepime, Cefiderocol, and inhibitor combinations) still need to be specified individually.
 - This maps roughly to the current refgene subclasses (which includes groups: beta-lactam, carbapenem, cephalosporin; plus some individual drugs, currently ceftaroline, cefiderocol, inhibitor-combinations).
 - CARD ARO currently does not currently distinguish different generations of cephalosporins, therefore the ontology is not yet suitable for the desired purpose here.

Category

Indicates the categorization associated with this gene, for members of this species {wt S, nwt S, wt R, nwt R, wt I, nwt I}. Wildtype/non-wildtype (wt/nwt) status should be determined based on

core gene status. S/I/R should be defined using EUCAST breakpoints. If the drug this rule applies to appears on the [Expected Resistances](#) list for this organism, and the gene is a core gene, the expected categorization should be 'wt R'. If the gene is identified as a core gene but the drug does not appear on the Expected Resistances list for this organism, there should be strong evidence from literature and/or matched genome/phenotype data to support the assignment of 'wt S' vs 'wt I' vs 'wt R'.

PMID

[PubMed](#) identifier for the 'best' peer-reviewed research article that provides evidence that this gene is associated with this phenotype category in this species.

Where a rule is made based on new unpublished data analysis, the supporting evidence should be submitted for review by the working group, and preprinted or published as soon as possible.

Future considerations:

- Would be useful to establish evidence codes, or confidence levels, to be associated with rule sets. This would support a range of use cases, including allowing filtering to high-confidence rules where desired, and distinguishing high vs low confidence in downstream reports or inferred antibiograms.

Rule curation note

A short explanatory note describing the mechanism and/or reasoning for the rule. Note this is intended to document the justification for setting the rule, not necessarily something to propagate into annotation of downstream genotype reports or genome reports.

Additional fields - in development

Drug class

The name of the drug class that the rule applies to. This field should be completed ONLY IF there is evidence that the gene has activity against all drugs in the class.

The reference for drug names and drug classes is the [Antibiotic Resistance Ontology](#) (part of [CARD](#)). [Cephalosporins](#) should be further differentiated into early (1st-2nd generation), extended-spectrum (3rd-generation), 4th generation, 5th generation, and siderophore cephalosporin.

Evidence level

Indicates the level of supporting evidence for a rule, to facilitate filtering on confidence of the interpretation. Would need a specification of evidence levels.

A key advantage is this would facilitate adding of rules on the basis of evidence from related organisms, or drugs in the same drug class, for which there is no direct species/drug/gene evidence. It would also facilitate and create clarity around adding of rules on the basis of

unpublished genome vs phenotype data available to the rule curators but not yet public, as these could be flagged and also indicate the strength of the evidence.

Breakpoint_standard

The AST phenotyping standard used to interpret this rule. In the format '[Name] [version] ([year])', e.g. 'EUCAST v14.0 (2024)' or 'EUCAST ECOFF (January 2024)' (as EUCAST [ECOFFs](#) are not versioned, indicate month and year). This is to facilitate including different interpretations using different AST standards, such as EUCAST, CLSI, veterinary standards, ECOFFs, etc and for clarity as to the definition used as breakpoints can change over time.

If it is an Expected (intrinsic) resistance, there will not typically be a breakpoint, in this case indicate the version of the expert rules e.g. 'Expected resistant phenotypes v 1.2 (13 January, 2023)'

Breakpoint

The breakpoint (preferably MIC) that was used to define the expected phenotype category. For categorization as 'R', breakpoint should be given in the form 'MIC >X [units]'; for categorization as 'S', use 'MIC <=X [units]'; for categorization as 'I', use 'I >X [units]'. This is to provide clarity as to the definition used, as breakpoints can differ between standards and change over time.

Alternative gene identifiers

This is proposed as it is useful for interoperability, to facilitate annotating output of tools that use other databases. There is no guarantee that any given tool will use any of these identifiers, and so this issue may be better addressed through a separate identifier harmonization effort; however Refseq and ARO are the most widely used primary databases so likely to support widespread interoperability:

Refseq accession: Refseq protein accession (for an allele) or HMM accession (for an internal node).

ARO accession: Antibiotic Resistance Ontology (ARO) identifier for the gene this rule applies to. Facilitates interoperability with tools that utilise ARO as the primary database rather than refgene. May become essential if ARO develop a gene-drug dictionary that AMRules would benefit from utilizing. Note there will not always be an ARO for every allele and node in refgene. In this case a representative may be chosen, or the field left blank.

Explanation

Several people have raised the need for a more clinically or epidemiologically informative 'note' field that would be suitable to propagate into a downstream genome report designed for clinical or public health knowledge users, e.g. flagging clinical considerations such as when the presence of an intrinsic beta-lactamase does not usually imply carbapenem resistance, but clinicians should be made aware that upregulation or copy number increases can occur that would result in carbapenem resistance. (Note the 'rule curation note' field is not intended for this purpose, but is rather intended to document the reasoning behind the rule.)

Rule set development

Drafting of rule sets

It is recommended that a copy of the rule [template](#) be created for each organism, to be shared by working group members who are working together on that specific organism. The mode of collaboration between members assigned to the same organism is left to the individual members, but we suggest creating a directory in the [AMRRulesCuration](#) GitHub repository for each organism to share files.

Key literature supporting each rule should be recorded in the 'PMID' field and a short description of the logic for each rule added to the 'note' field of the rule template. In addition, evidence for rules should be documented in a short narrative (e.g. in a Google doc), explaining the logic followed and any data analysis undertaken to develop and test the rules. Members may wish to consider publishing this narrative, either stand-alone or as part of umbrella article/s describing the project; this will be discussed at the working group progress meetings.

Review of rule sets

Once a rule set is ready for review, the file (in TSV format) should be added to the [/drafrules](#) directory in the [AMRRulesCuration](#) GitHub repository. The working group Chair/s will review them for format and content (including the linked supporting evidence), and the group's Lead Bioinformatician will review and test using available wildtype and non-wildtype genome data. These test data will form part of a test suite (hosted in a separate repository) to be included in the main paper describing development and testing of the interpretive rules. Criteria for review and testing will be reviewed and discussed as the working group progresses.

Release of approved rule sets

Once reviewed and approved, new rule sets will be added to the master rule set in the main branch of the [AMRRules github repository](#), and a versioned release will be made that includes the new rules together with release notes recording the addition of the organism and the names of all contributors.

NOTE: As we aim for AMR rules to be widely adopted, it may be necessary to add steps to the review and release cycle to meet the needs of stakeholder organizations such as CARD or EUCAST.

Initial Protocol: Wildtype phenotypes

This protocol should be considered a starting point for the working group, and will be developed and refined as the group attempts to populate rules.

This protocol focuses on wildtype phenotypes, as the first priority of the working group. Where sufficient data is available to propose rules for acquired resistance, these may be recorded. However such rules will not be included in the first release of the AMR rules, as inclusion criteria and standards of evidence need to be defined first.

Wildtype resistant

1. Add a row for each drug on the EUCAST '[Expected resistance](#)' list for the organism

- Make sure to use the right (generic, not brand) name for the drug, this should be the name of the drug as it appears in the Expected Resistance list, but please check this matches exactly an entry in the [ARO](#). If it does not, flag for review.

2. If you already know the gene/s responsible for a trait, then:

- Confirm whether the gene meets the definition of 'core', and complete the 'context' field.
- Locate the gene/alleles in [refgene](#) to find the corresponding node ID/s to enter in the 'gene' field.
- Review whether there is a suitable internal node in the [reference gene hierarchy](#) (e.g. for *fosA* in *Klebsiella pneumoniae*, the wildtype chromosomal alleles correspond to *fosA5*, *fosA6*, *fosA9*, *fosA10* which descend from the common ancestor node *fosA5_fam*, therefore the correct node to specify this rule is '[fosA5_fam](#)'). If it is not clear which alleles/nodes capture the right gene, flag that additional analysis will be needed to resolve this. If there is no single node to describe the rule, add separate rules for each node to which you think it applies (one row for each).
- Review the supporting literature to find those articles that provide the best evidence for this gene being responsible for wildtype (intrinsic) resistance to the drug in this organism.
- If you are sure there is sufficient evidence that this specific gene supports the interpretation of 'wt R' using current breakpoints, record this as the interpretation. If there is any uncertainty, flag this for review.
- Record the [PubMed](#) identifiers for the relevant citations in the PMID column. If possible try to add one citation per unique node, however if the same citation supports multiple alleles/nodes that is permissible.
- Write a short explanatory 'note' if needed to support the interpretation and explain any points of potential confusion.

3. If you don't know the core gene responsible:

i) Conduct some literature searches to check if this is known;

ii) Check the 'Existing Curations' resources below; and

iii) Review the output of AMRfinderplus on genomes from 'wildtype' organisms

(susceptible to all drugs besides those on 'Expected resistance' list) to try to identify 'intrinsic' determinants associated with each expected resistance.

- This depends on availability of genome data with matched antibiogram data.
 - **Public AST data** can be found via [NCBI Pathogens AST Browser](#) and [BV-BRC](#).
 - **Consistently assembled public genomes** for a given species can be easily downloaded as a single tarball from the [AllTheBacteria](#) FTP site (described [here](#)). Note that their team is in the process of running AMRFinderplus on all genomes, check the [github](#) for progress which may save you running AMRFinderplus yourself.
 - **Note that it is not recommended to rely on the genotype calls in the NCBI Pathogens browser, as these calls are currently not re-run with new updates of AMRFinderplus, so the results include calls from a mix of versions and differences between genomes may reflect differences in the version that was run rather than differences in the actual genotypes.**
 - If downloading assemblies from other sources, species should be confirmed by reference to GTDB, using [GTDB-Tk](#).
 - Consideration should be given to the quality and source of genome data and AST data, and the diversity of the genome data.
 - A suggested rule of thumb to define a gene as a core gene candidate for expected resistance is n=50 genomes with wildtype antibiogram, including >20 unique lineages (defined by unique MLST lineage, or clustering based on mash distance). Ideally the isolates should also be diverse in terms of geography and source. Specific criteria will be developed by the working group as we go, please just try to maximize the diversity of data you include and be sure to record details of these parameters for review and discussion later.
 - MICs based on microbroth dilution are the preferred source of AST data. If you need to combine multiple sources of data from different laboratories, instruments or methods take care to compare the MIC distributions and check for batch effects.
 - If you don't have enough genomes with known wildtype antibiograms, it may be useful to explore all genomes (regardless of antibiogram availability or result) in order to identify core genes that may explain expected resistance.
 - AMRfinderplus should be run afresh on the genome assemblies, using the latest version and parameters (including the organism option, if this organism [is supported by AMRFinderPlus](#)).
- Example command:
- ```
amrfinder -n genomeA.fasta --plus --print_node --organism
Acinetobacter_baumannii --name genomeA > genomeA_amr_results.txt
```
- **(Note the values in the 'AMR genotypes' field in NCBI Pathogens online portal cannot be relied upon for our purposes, as it is not rerun each time the databases are updated, and so there can be inconsistencies in the outputs for different genomes that are added years apart.)**
  - Map the detected core determinants to the Expected resistances using refgene class/subclass and PMIDs (in the AMRfinderplus output) and your own literature searches.
  - Complete the rules, one per gene/node, as outlined above.

**4. If you still have unexplained Expected resistances**, i.e. where you can't identify a core gene responsible in the AMRfinderplus, some options for further exploration of potential mechanisms are:

- [ResFam](#) search of wildtype genomes
- [CARD RGI](#) search of wildtype genomes

### Wildtype susceptible

**1. Review the output of AMRfinderplus** on a set of organisms with 'wildtype' antibiograms (i.e. no resistances beyond drugs with Expected resistance). See above for where to find genomes with matched AST data and to run AMRfinderplus.

**2. If intrinsic determinants are reported by AMRfinder that don't map to an Expected resistance, these may require a 'wt S' rule.** It is unlikely they reflect true 'wt R' that is not included in the Expected resistance.

- First confirm the gene is core, across a diverse set of genomes, considering those with antibiograms as well as the broader population.
- For those with antibiograms, examine the MIC distribution for isolates whose genomes have no other known determinants for this drug. Compare this with the clinical [breakpoints](#) to confirm it's clear the wildtype phenotype is susceptible.
- Review the reference MIC/DD distributions at [EUCAST](#). Is the organism's wildtype distribution shifted relative to close neighbours? I.e. Does it have a higher ECOFF? (e.g. fosfomycin wildtype distribution for *Klebsiella pneumoniae* is higher than for *E. coli* due to core gene *fosA*).

**3. Review literature evidence** for the gene's link to resistance and associated mechanism

- Is there any evidence for this specific organism, or is the evidence for resistance in other organisms only?
- Is its resistance linked to mobile elements in those other organisms?

**4. If these steps identify no evidence of resistance**, record the interpretation as wt S and add the relevant supporting PMIDs and notes.

## Open issues

### Which drugs

should be considered/included for a given organism?

- If [Expected resistance](#) is recorded for drug X, the rule set should aim to include and provide interpretation for the core gene/s responsible for this resistance.
- If a gene is associated with a large drug class, consideration should be given as to which drugs in that class are relevant to include individual rules for. The existence of EUCAST clinical breakpoints is a good indication of whether the drug is relevant to treatment. Priority should also be given to drugs recommended for global surveillance by [WHO GLASS](#), and those on the [WHO Global Essential Medicines List](#) (or national lists and treatment guidance).

### Defining resistance

#### Multiple breakpoints

If there are multiple breakpoints for the same drug-bug combination for different clinical conditions? E.g. for Enterobacterales, the ciprofloxacin R breakpoint is >0.125 for meningitis, and R >0.5 for indications other than meningitis.

- Specify a separate rule for each breakpoint, indicating the breakpoint in the 'breakpoint' field (e.g. 'MIC >0.125 mg/L') and the indication in the 'note' field (e.g. 'breakpoint for meningitis').

#### No breakpoint?

- Consider any relevant notes in the [EUCAST Breakpoints Table](#), consult EUCAST [guidance](#) documents, and check whether the organism has [Expected Resistance](#).
- Note that if EUCAST Breakpoints Table has a 'dash' instead of numerical values it indicates the microbe can be reported resistant without further testing. The same is true if Expected Resistance is recorded for this drug in this organism. If either or both of these is true, it implies the interpretation should be 'wt R' IF there is clear evidence that the wildtype resistance is functionally attributable to this specific gene.
- If the above do not apply (ie there is no evidence of wildtype resistance) but there is an ECOFF, consider specifying a rule based on the ECOFF instead. Indicate the ECOFF in the 'breakpoint' field, and record 'ECOFF [date]' in the 'breakpoint\_standard'. If a gene does not push the MIC distribution above the ECOFF the correct categorization is 'wt S'. If the gene pushes the MIC distribution above the ECOFF but there are no breakpoints, the correct categorization is 'nwt'; it cannot be 'nwt R' as there is no definition of R.
- In the absence of an ECOFF or any other guidance, it may be suitable to define an unofficial (internal-use) ECOFF based on available MIC distributions and interpret against this; this would need to be documented in the 'note' column and fully described

in the narrative description (including showing the distribution, and the source of the data).

### MIC distribution vs breakpoint

What to do if a genetic determinant shifts an MIC distribution upwards, but not so high that the tail of the distribution exceeds a breakpoint or ECOFF? What if the breakpoint cuts the MIC distribution in half? What fraction of the distribution needs to exceed an 'R' breakpoint to interpret the gene as 'nwt R'?

Note this generally relates to the effect of **acquired** resistance determinants, as opposed to resolving the effect of core genes and understanding wildtype phenotypes, which is Phase 1 and the priority for the ESGEM-AMR working group. Resolving these issues conclusively will require large amounts of high-quality AST data with matched genome data, as part of Phase 2.

There are inherent difficulties in creating rules based on the effect of a gene on MIC, as what we observe is a distribution of MIC values not a single value. Sources of variation include natural biological variation, and technical variation in measuring the MIC. Notably, MICs are measured via doubling dilutions, they are not measured as continuous variables, so the underlying distribution is not directly observed. Ideally, MIC distributions would be bimodal with two peaks corresponding to wildtype susceptible (responsive to drug treatment) and nonwildtype resistant (likely to fail treatment even on high exposure), and breakpoints would be set such that they fall in clear troughs between those two peaks. However the reality is not always so clear, particularly as we are observing the distribution of discretised measures of MIC rather than the underlying distribution.

In principle, a rule defining the expected impact of a gene X on a MIC category for drug Y (e.g. gene X => nwt R) could be defined in a number of ways, with respect to how the breakpoint relates to the observed MIC distribution (for isolates that carry gene X but no other known acquired determinants for drug Y). Possible rules could be:

1. Any of the distribution exceeds the breakpoint;
2. The expected value exceeds the breakpoint (in the statistical sense of the expected value of a random variable, which equals the mean value), or
3. The majority (or entirety) of the distribution exceeds the breakpoint.

In practice, requiring the entirety of the distribution exceed a breakpoint is impractical as there is likely to be a lower tail of the observed MIC distribution that falls below the breakpoint due to technical artefacts (e.g. assay variability, errors in the sequence data, or changes during culture steps between AST and DNA extraction for sequencing). Basing a rule on the upper extreme of the distribution is also problematic as this too could be driven by technical artefacts, or by rare unknown resistance determinants.

Requiring a fraction of the distribution exceed the breakpoint avoids the issue of extreme values in the tails of the MIC distribution. The question then becomes what fraction. A required fraction of half or greater is equivalent to requiring that the expected value exceeds the breakpoint. This



is attractive as it is simple to define and does not require parametrization of the distribution, which is problematic given the non-continuous nature of MIC data. A pragmatic solution is therefore to require only half the observed values to exceed the breakpoint in order to define the phenotype category, but to record details of the observed fraction as an indicator of confidence in the categorization. **In practice, it is expected that investigative analyses of real, large, MIC distributions for multiple drugs and bugs in Phase 2 will help bring clarity to this issue and facilitate the definition of clear guidance and confidence metrics for interpretation of acquired genes.**

- The proposed rule of thumb is to set rules based on the expected value of the MIC associated with the gene (ie >half the distribution exceeds the breakpoint).
  - If the median MIC of isolates carrying gene X and no other known determinants of Y exceeds the R breakpoint, the interpretation is 'nwt R'.
  - If the median MIC exceeds the S breakpoint but not the R breakpoint, then the categorization is 'nwt I'.
  - If the median MIC exceeds the ECOFF but not the S breakpoint, the categorization is 'nwt S'.
  - If the median MIC falls below the ECOFF the categorization is 'wt S'.
- The 'note' field can be used to record when the rule is borderline, e.g. if the fraction of the distribution exceeding the breakpoint is less than 90%.

## Combinatorial rules

How to capture combinatorial rules, ie where a combination of determinants is required to achieve a phenotype?

**Notably, this mainly relates to categorizing the effect of **acquired** resistance determinants (as opposed to resolving the effect of **core** genes and understanding wildtype phenotypes, which is the first priority for the working group) and will require large amounts of high-quality AST data with matched genome data to properly define.**

## General principles

- The additive assumption should hold unless specified otherwise; i.e.
  - If there is a rule 'gene1 => nwt R for drug X' this is assumed to hold regardless of other rules specified for drug X, or for any other drug.
  - It is assumed that resistance to combined drug X+Y requires resistance to drug X and drug Y. If there are rules 'gene1 => nwt R for drug X' and 'gene2 => nwt R for drug Y', resistance to combined drug X+Y is assumed.

## Combination drugs

- For simple drug combinations such as trimethoprim-sulfamethoxazole, the assumption is that resistance requires a determinant conferring resistance to each component (e.g. a determinant conferring trimethoprim resistance plus a determinant conferring sulfamethoxazole resistance). Therefore, separate rules should be provided for each

component drug, and inference of resistance to the combination can be trivially made by checking for resistance to both component drugs.

- If a gene were identified that conferred resistance to the combination drug, AND each of its constituent components individually, this could be handled by indicating two rules, one for each component drug (e.g. gene => trimethoprim R and one for gene => sulfamethoxazole R). Again, resistance to the combination can be trivially inferred from resistance to both component drugs.
- If a gene were identified that conferred resistance to the combination drug and JUST ONE OF its constituent components, this could be handled by indicating two rules, one for gene => combination and one for gene => [component drug].

### Drug + inhibitor combinations

- In some cases, resistance is conferred by a single gene, e.g. a beta-lactamase that evades inhibition to hydrolyze the drug in the presence of drug+inhibitor. The correct way to specify this is a single rule specifying 'gene => drug+inhibitor nwt R'.
- If separate resistance determinants are known for the drug and the inhibitor, the assumption is that these work additively such that presence of both confers resistance to the combination. In this case each 'gene => drug' or 'gene => inhibitor' rule should be specified separately.
- Sometimes separate resistance determinants are known for the drug and the inhibitor, but these do not always work additively, e.g.:
  - 'gene1 => drug nwt R'
  - 'gene2 => drug nwt R'
  - 'gene3 => inhibitor nwt R'
  - 'gene1+gene3 => drug+inhibitor wt S'
  - 'gene2+gene3 => drug+inhibitor nwt R'.
- In this case, the individual rules should be specified along with any **exceptions** to the additive assumption, i.e.:
  - 'gene1+gene3 => drug+inhibitor wt S' should be specified, as it differs from the additive effect assumed from the individual gene1 and gene3 rules (which would be 'drug+inhibitor wt R')
  - It is not essential to specify a rule that 'gene2+gene3 => drug+inhibitor nwt R' as this is the assumption from the rules 'gene2 => drug nwt R' and 'gene3 => inhibitor nwt R', although it may be helpful to make this explicit and provide evidence.

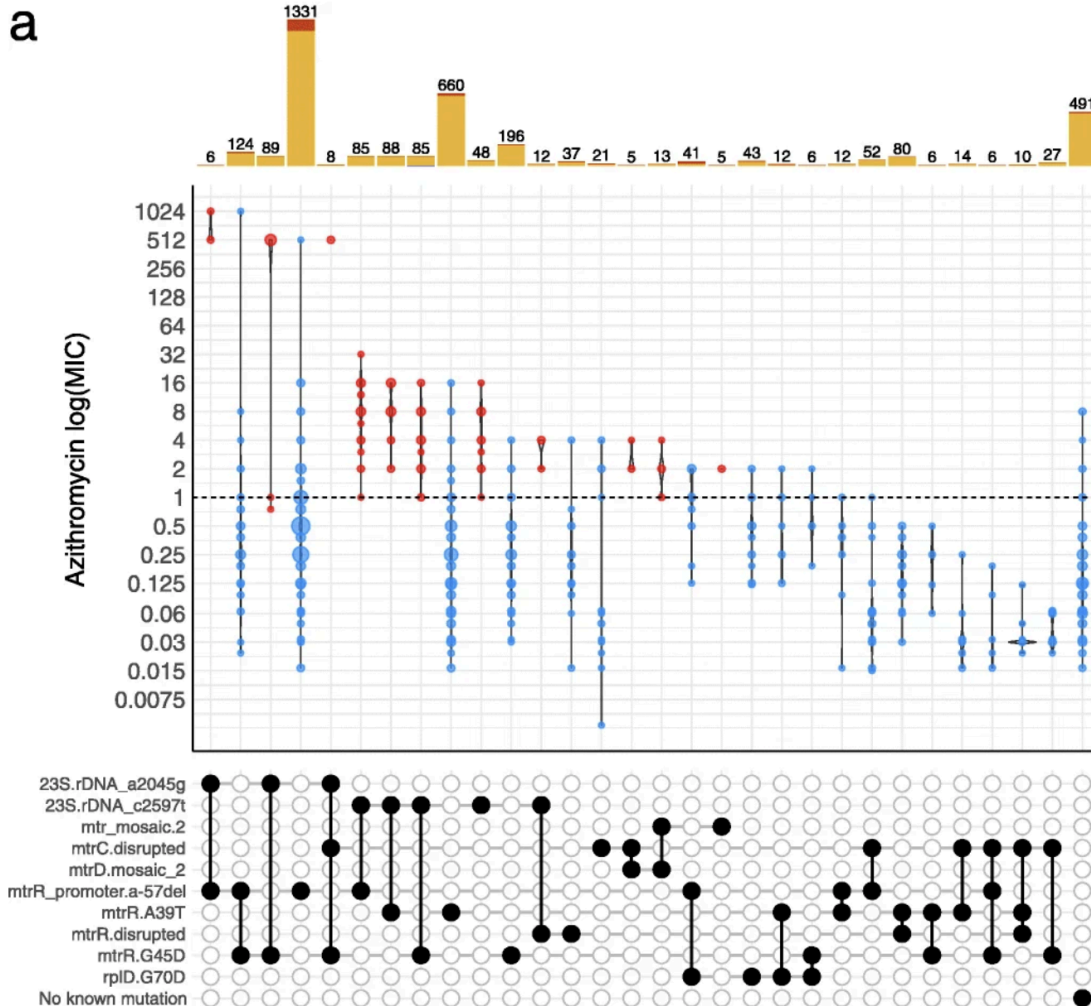
### Single drug, multiple determinants

Some genes have a small or no effect on their own, but in combination can shift an MIC substantially.

- If the combined effects are simply additive (e.g. 'gene1 => wt S', 'gene2 => nwt R', 'gene1+gene2 => nwt R') then there is no need to specify a combinatorial rule.
- If the combined effect is not additive ('gene1 => wt S', 'gene2 => wt S', 'gene1+gene2 => nwt R') then a separate rule should be given for the combination.

- Note that to minimise the need for specifying large numbers of combinatorial rules, curators should attempt to identify an internal node in the gene hierarchy that captures the rule accurately, rather than specifying each individual allele. E.g. a single rule 'GyrA\_S83F + qnr => ciprofloxacin nwt R' is preferable to multiple rules for 'GyrA\_S83F + qnrS1', 'GyrA\_S83F + qnrB1', 'GyrA\_S83F + qnrB2' (assuming of course this specification is accurate).

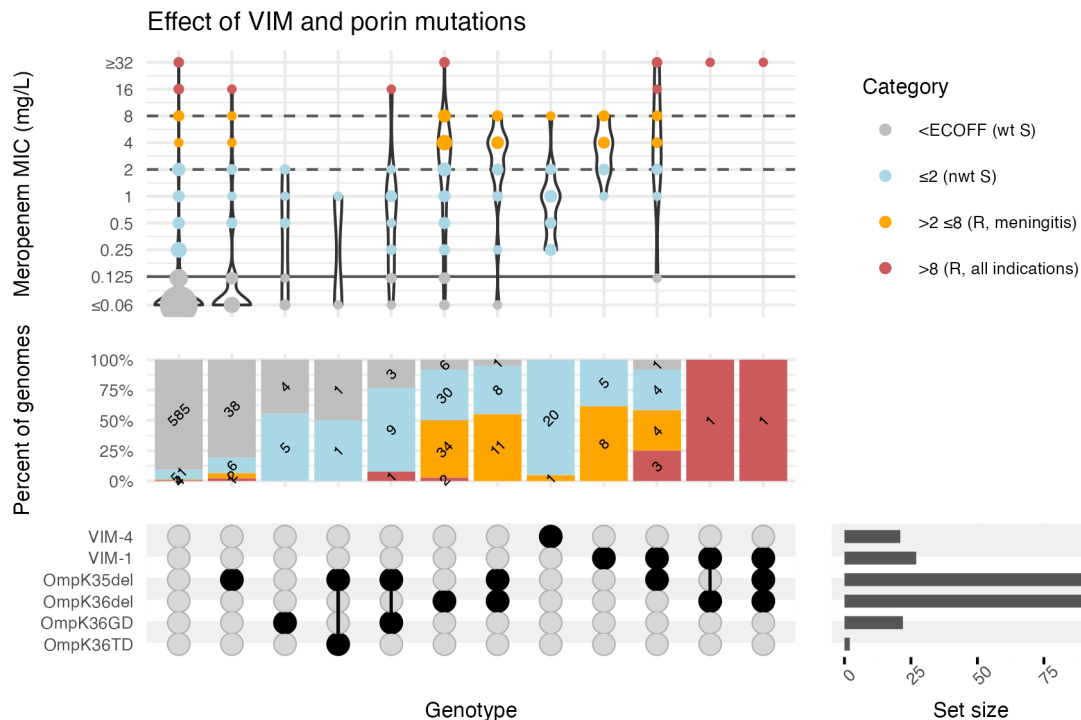
**Example 1: resistance in *N. gonorrhoeae*** is mostly mutational, and combinations are often required and need to be specified individually, see e.g. [Figure 3 here](#) (reproduced below):



**Example 2: *van* operon**, which includes several genes, including multiple that are required for vancomycin resistance in e.g. [Enterococcus faecium](#) and multiple that aren't. Therefore presence of a single gene is not sufficient to define a rule set, and rules would need to specify the combination/s that are known to reliably result in resistance (and potentially specify those that are known to retain susceptibility, for clarity).

### Example 3: combination of carbapenemase enzymes with porin mutations.

Some carbapenemases (e.g. VIM and OXA variants) raise meropenem MIC in *Klebsiella pneumoniae* above the ECOFF (0.125 mg/L), but not above the S breakpoint (2 mg/L). In combination with porin mutations the MIC distributions shift further upwards, sometimes above the S/R breakpoint for meningitis (R >2) and sometimes above the R breakpoint for other indications (R >8). See figure below, which is produced from the meropenem MIC data from [EUSCAPE](#) mapped to carbapenemase and porin mutations called by [Kleborate](#) in the corresponding genomes.



- In this case, following the guidance above and based on the most common effect shown in the plot, we would need to specify:
  - ompK35-deletion => **wt S**
  - ompK36GD => above ECOFF but below S => **nwt S**
  - ompK36-deletion => above S (2) but below R (8)  
=> **nwt R** for meningitis, **nwt I** for other indications
  - VIM-4 => above ECOFF but below S => **nwt S**
  - VIM-1 => above S (2) but below R (8)  
=> **nwt R** for meningitis, **nwt I** for other indications
  - VIM-1 + ompK36-deletion => above R (8)  
=> **nwt R** for all indications
  - Combinations with ompK35-deletion + ompK36-deletion don't need to be specified because they obey the additive assumption
  - Some combinations are not observed so cannot be defined from this data
- Note that carbapenemases that raise MIC >8 in the absence of any porin mutations (such as KPC, NDM, IMP) only require a single rule to assign 'wt R' for each indication.

## Existing Curations

### AbritAMR enhanced drug class/subclass

Copy of refgenes database with 'enhanced\_class' and 'enhanced\_subclass' appended  
[https://github.com/MDU-PHL/abritamr/blob/master/refgenes\\_2024-02-23.csv](https://github.com/MDU-PHL/abritamr/blob/master/refgenes_2024-02-23.csv)

### ResFinder phenotype resources

ResFinder has undertaken curation of genes and mutations to drugs, for selected organisms.

#### List of in-silico panels used in ResFinder (list of drugs covered per organism):

[https://bitbucket.org/genomicepidemiology/resfinder\\_db/src/master/phenotype\\_panels.txt](https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/phenotype_panels.txt)

- Campylobacter
- Campylobacter jejuni
- Campylobacter coli
- Enterococcus faecalis
- Enterococcus faecium
- Escherichia coli
- Mycobacterium tuberculosis
- Salmonella
- Salmonella enterica
- Staphylococcus aureus

#### Mapping of drug to class:

[https://bitbucket.org/genomicepidemiology/resfinder\\_db/src/master/antibiotic\\_classes.txt](https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/antibiotic_classes.txt)

#### Phenotype panels (mapping gene to drug, not organism specific):

[https://bitbucket.org/genomicepidemiology/resfinder\\_db/src/master/phenotypes.txt](https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/phenotypes.txt)

Example rows:

| Gene_accession no.      | Class                     | Phenotype                                                                              | PMID                         | Mechanism of resistance | Notes                                                                                                                                                           |
|-------------------------|---------------------------|----------------------------------------------------------------------------------------|------------------------------|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| aac(6)-Ib-cr_1_DQ303918 | Aminoglycoside, Quinolone | Tobramycin, Dibekacin, Amikacin, Sisomicin, Netilmicin, Fluoroquinolone, Ciprofloxacin | unpublished                  | Enzymatic modification  | MIC of ciprofloxacin does not always increase above ECOFF PMID 16369542                                                                                         |
| blaOXA-51_1_DQ385606    | Beta-lactam               | Amoxicillin, Ampicillin, Imipenem                                                      | 16940137, 15649299, 16630258 | Enzymatic inactivation  | Class D; OXA-51-like; Natural in Acinetobacter baumannii;; Phenotype described in Acinetobacter baumannii if simultaneous presence of insertion sequence ISAba1 |
| blaOXA-66_1_AY750909    | Beta-lactam               | Unknown Beta-lactam                                                                    | 15760431                     | Enzymatic inactivation  | Class D; OXA-51-like; Natural in Acinetobacter baumannii;;                                                                                                      |

## Phenotype panels (mapping mutation to drug):

[https://bitbucket.org/genomicepidemiology/pointfinder\\_db/src/master/campylobacter/phenotypes.txt](https://bitbucket.org/genomicepidemiology/pointfinder_db/src/master/campylobacter/phenotypes.txt)

Organisms with mutations in DB:

- Campylobacter
- Enterococcus faecalis
- Enterococcus faecium
- Escherichia coli
- Helicobacter pylori
- Klebsiella
- Mycobacterium tuberculosis
- Neisseria gonorrhoeae
- Salmonella
- Staphylococcus aureus

Example rows for Klebsiella:

| #Gene_accession   | TypeGene | Mutation ID | Codon_pos | Ref_nuc | Ref_codon | Res_codon               | Class        | Phenotype                     | PMID     | Mechanism of resistance | Notes                                                     |
|-------------------|----------|-------------|-----------|---------|-----------|-------------------------|--------------|-------------------------------|----------|-------------------------|-----------------------------------------------------------|
| ompK36_1_Z33506.1 | AA       | ASP-49      | 49        | GAC     | D         | S                       |              | Cephalosporins                | 25245001 |                         | Under development. Phenotype should be used with caution. |
| acrR_1_AJ318073.1 | AA       | ALA-20      | 20        | GCT     | A         | T                       | Quinolone    | Fluoroquinolone               | 12936981 |                         | Under development. Phenotype should be used with caution. |
| ramR_1_KY465996.1 | AA       | ALA-19      | 19        | GCG     | A         | V                       | Tetracycline | Tigecycline                   | 28533243 |                         | Under development. Phenotype should be used with caution. |
| gyrA_1_AF052258.1 | AA       | SER-83      | 83        | TCG     | S         | L                       | Quinolone    | Ciprofloxacin                 | 22633335 |                         | Under development. Phenotype should be used with caution. |
| ompK36_1_Z33506.1 | AA       |             | 131       | -       | ins       | SG                      |              | Cephalosporins                | 25245001 |                         | Under development. Phenotype should be used with caution. |
| acrR_1_AJ318073.1 | NUC      |             | 382       | -       | del       | CAGGC<br>CCAGC<br>GGCAG | Quinolone    | Norfloxacin,<br>Ciprofloxacin | 12936981 |                         | Under development. Phenotype should be used with caution. |

Example rows for Salmonella:

| #Gene_accession      | TypeGene | Mutation ID | Codon_pos | Ref_nuc | Ref_codon | Res_codon | Class          | Phenotype                        | PMID     | Mechanism of resistance |
|----------------------|----------|-------------|-----------|---------|-----------|-----------|----------------|----------------------------------|----------|-------------------------|
| pmrA_1_CP055130.1    | AA       | GLY-15      | 15        | GGG     | G         | R         | Polymyxin      | Colistin                         | #####    | Target modification     |
| pmrB_1_CP051284.1    | AA       | ARG-81      | 81        | CGC     | R         | C,H       | Polymyxin      | Colistin                         | #####    | Target modification     |
| gyrA_1_MH933946.1    | AA       | ALA-67      | 67        | GCC     | A         | P         | Quinolone      | Nalidixic acid,<br>Ciprofloxacin | 7492118  | Target modification     |
| acrB_1_CP000026.1    | AA       | ARG-717     | 717       | CGG     | R         | Q,L       | Macrolide      | Azithromycin                     | 31730615 | Target modification     |
| 16S-rsD_1_CP049983.1 | NUC      | CYS-1065    | 1065      | C       | C         | T         | Aminoglycoside | Spectinomycin                    | 12402084 | Target modification     |

## CARD Prevalence

When users search CARD for a specific gene/allele, the 'Resistomes' tab will display the prevalence of the gene per species, estimated from NCBI Chromosome, NCBI Plasmid, and NCBI WGS. This can be helpful to explore which organisms a gene is core to and to what extent it is plasmid-borne and disseminated to other organisms.

E.g. searching for [FosA6](#), which is intrinsic (S<sup>WT</sup>) in *K. pneumoniae* yields:

Prevalence: protein homolog model ([view sequences](#))

| Species                           | NCBI Chromosome | NCBI Plasmid | NCBI WGS |
|-----------------------------------|-----------------|--------------|----------|
| <i>Escherichia coli</i>           | 0%              | 0%           | 0.05%    |
| <i>Klebsiella aerogenes</i>       | 4%              | 0%           | 3.95%    |
| <i>Klebsiella pneumoniae</i>      | 84.97%          | 0.01%        | 50.11%   |
| <i>Klebsiella quasipneumoniae</i> | 91.6%           | 0%           | 68.03%   |
| <i>Salmonella enterica</i>        | 0%              | 0%           | 0.01%    |

E.g. searching for [FosA2](#), which is intrinsic in the *Enterobacter cloacae* complex:

Prevalence: protein homolog model ([view sequences](#))

| Species                          | NCBI Chromosome | NCBI Plasmid | NCBI WGS |
|----------------------------------|-----------------|--------------|----------|
| <i>Enterobacter asburiae</i>     | 87.1%           | 0%           | 64.82%   |
| <i>Enterobacter cancerogenus</i> | 83.33%          | 0%           | 85.71%   |
| <i>Enterobacter chengduensis</i> | 100%            | 0%           | 84%      |
| <i>Enterobacter cloacae</i>      | 92.86%          | 0%           | 64.22%   |
| <i>Enterobacter hormaechei</i>   | 87.05%          | 0%           | 58.83%   |
| <i>Enterobacter kobei</i>        | 90.91%          | 0%           | 63.76%   |
| <i>Enterobacter roggenkampii</i> | 69.77%          | 0%           | 52.88%   |
| <i>Escherichia coli</i>          | 0%              | 0%           | 0.01%    |
| <i>Klebsiella pneumoniae</i>     | 0%              | 0%           | 0%       |
| <i>Providencia stuartii</i>      | 62.5%           | 0%           | 50%      |
| <i>Salmonella enterica</i>       | 0.06%           | 0%           | 0.01%    |