

Computational Practical 6: Detecting antimicrobial resistance from bacterial genomes using command-line tools

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Introduction

Growing rates of antimicrobial resistance make antibiotic susceptibility testing (AST) increasingly needed to ensure the right antibiotics are prescribed for patients with bacterial infections. Determining antibiotic susceptibility is preferred over empiric therapy, wherein typically broad-spectrum drugs are used without a definitive confirmation of the infectious agent and which antibiotics infectious bacteria are resistant to. Data collected on antibiograms (strains' full susceptibility pattern) can also be used for surveillance purposes and, in turn, inform empiric therapy.

AST is routinely performed using culture-based techniques in clinical diagnostic laboratories, frequently disk diffusion, broth microdilution and gradient diffusion (i.e. E-test). As antibiotic resistance is genetically encoded, i.e. mediated by acquisition of new genes, gene copy number, and mutations in regulatory and coding regions of existing chromosomal genes, molecular tests have been developed to target the detection of such genetic markers.¹ In the last decade, **whole-genome sequencing** has emerged as an alternative technology to both culture and targeted molecular tests for the detection of AMR as it can, in principle, detect all AMR genetic determinants and predict resistance to all antibiotics in a single experiment. The

accuracy of genotypic predictions depends on the availability of: (1) accurate databases of AMR genetic determinants, (2) large collections of whole-genome sequenced strains with AST measurements to assess the diagnostic accuracy of such catalogues, and (3) automated genome analysis and interpretation tools.

Mutational (chromosomal) resistance is the main driver of acquired resistance in certain bacterial species, such as *Mycobacterium tuberculosis* and *Helicobacter pylori*, or for particular antibiotics, especially to synthetic agents such as fluoroquinolones and oxazolidinones. Resistance mutations are vertically transmitted, i.e. via clonal reproduction of bacteria, or can be transmitted horizontally via homologous recombination between different strains. **Gene-mediated resistance** is the main driver of acquired resistance in certain bacterial species, particularly gram-negatives. Resistance genes can be horizontally transmitted (via vectors such as plasmids) and also vertically transmitted (particularly stable if integrated into the chromosome). In some bacterial species, chromosomal and gene-mediated resistance are equally common (e.g. *Staphylococcus aureus*). Resistance to the same antibiotic can be conferred by both mutations and acquired genes (e.g. fusidic acid in *Staphylococcus aureus*, colistin resistance in *Escherichia coli*).

Over the years, a number of global studies have identified the genes and mutations that confer resistance to particular antibiotics. There are several databases such as the **Comprehensive Antimicrobial Resistance Database (CARD)** (<https://card.mcmaster.ca/>), **ResFinder** (<https://cge.cbs.dtu.dk/services/ResFinder/>), **AMRFinder** (<https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/>) or **Pathogenwatch** (<https://pathogen.watch/>) that contain information about the genes and mutations that confer resistance. The use of these databases and tools depends on the species and mechanisms of resistance one is interested in. There are few other resources that are specific to particular bacterial species, for example Mykrobe (<https://www.mykrobe.com/>) can be used to detect AMR in *Mycobacterium tuberculosis* (*M. tb*) and *Staphylococcus aureus* and TB Profiler (<https://tbdr.lshtm.ac.uk/>) which can be used for *M. tb* only.

6.1 Bacterial strains to be analysed

Table 6.1 contains the list of strains to be analysed in this practical. We will use three different command-based tools (AMRFinder, ResFinder and CARD RGI) to identify AMR genetic determinants from whole-genome sequences (from raw sequencing reads and genome assemblies). These strains were sourced from key studies on the genomic epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA)² and extensively drug-resistant (XDR) *Salmonella typhi*.³ In later computational practicals we will explore the epidemiology of outbreaks and epidemic clones of these bacteria, and study the strains investigated in this practical in a broader context. We will also

identify the genomic context of AMR genes, for example, if they are carried on mobile genetic elements.

Table 6.1

Species	Study	Strain Id	Genome accession	Assembly file name	Origin
<i>S. aureus</i>	holden2013	H65	ERR038057	holden2013_H65.fa	Oxford, England (1997) – ST22 MSSA
	holden2013	07-02477	ERR017261	holden2013_07-02477.fa	Berlin, Germany (2007)- ST22 EMRSA-15
	holden2013 (Artemis)	HO50960412	HE681097 (GenBank)	HO50960412.fa	UK (2005) - ST22 EMRSA-15
<i>S. typhi</i>	klemm2018 (ACT)	BL0006	ERR2093245	BL60006.fa	Pakistan (2016) – 4.3.1 (H58) XDR
	parkhill2001 (ACT)	CT18	AL513382 (GenBank)	CT18.fa	Vietnam (1993) - MDR
	klemm2018	Pak60168	ERR2093329	to be added	Pakistan (2016) – 4.3.1 (H58) pre-XDR

6.2 WGS-based prediction of AMR using ResFinder

6.2.1 Introduction to ResFinder

ResFinder, developed by Center for Genomic Epidemiology at the Technical University of Denmark (<http://www.genomicepidemiology.org/>), is a freely accessible tool to identify acquired genes and/or chromosomal mutations mediating antimicrobial resistance in total or partial DNA sequence of bacteria. Published in 2012 for the first time⁴, ResFinder was the first web-based bioinformatics tool developed to provide detection of AMR genes in WGS, aimed at users without specialized bioinformatic skills. A command-line (<https://bitbucket.org/genomicepidemiology/resfinder/>) version was later developed which allows the automation of ResFinder analyses within bioinformatic scripts. The authors claim ResFinder (web-based version, September 2021) has been executed

more than 800,000 times from more than 61,000 different users in over 171 countries.⁵

ResFinder, originally developed to detect acquired AMR genes, was later expanded with PointFinder⁶, a tool that detects chromosomal point mutations mediating resistance to selected antimicrobial agents. Recently, additional databases were developed to link each AMR determinant with phenotypic resistance to specific antimicrobial compounds, and species-specific panels for *in silico* antibiograms. ResFinder 4.0 was validated for several bacterial species including *Salmonella* spp. and *Staphylococcus aureus* strains with a diversity of AST profiles, human and animal sources and geographical origins.⁷

6.2.1 ResFinder commands

Navigate to the 'cp6' directory:

```
cd ~/course/cp6/
```

The raw sequencing reads and genome assemblies used in this practical can be found in the directory `~/course/cp6/`

Because the genomes of our strains are available as raw sequencing reads or genome assemblies, we will need to indicate ResFinder the format of input files. ResFinder can analyse both paired-end Illumina reads in fastq.gz format and genome assemblies in FASTA format.

Execute the command below to display all ResFinder arguments and options:

```
python -m resfinder --help
```

Next, download a local copy of the latest ResFinder databases:

```
git clone https://bitbucket.org/genomicepidemiology/resfinder_db/  
git clone https://bitbucket.org/genomicepidemiology/pointfinder_db/  
git clone https://bitbucket.org/genomicepidemiology/disinfinder_db/
```

Local databases need to be indexed using kma:

```
cd resfinder_db  
python3 INSTALL.py kma_index  
cd ..  
cd pointfinder_db  
python3 INSTALL.py kma_index  
cd ..  
cd disinfinder_db  
python3 INSTALL.py kma_index
```

Set approximate environment bash variables for ResFinder executable to locate these databases.

```
export CGE_RESFINDER_RESGENE_DB="/xxx/cp6/resfinder_db"  
export CGE_RESFINDER_RESPOINT_DB="/xxx/cp6/pointfinder_db"  
export CGE_DISINFINDER_DB="/xxx/cp6/disinfinder_db"
```

Remember to set these variables in any new terminal window. Otherwise ResFinder will exist with the error: 'Could not locate ResFinder database path'.

Now everything is set to run ResFinder on your terminal screen as shown in the command below:

```
python -m resfinder -ifa HO50960412.fa -s "Staphylococcus aureus" --acquired  
--point --outputPath HO50960412_resfinder
```

The command line above was used to run ResFinder on the genome assembly of *S. aureus* HO50960412 strain (Table 1). Note the following parameters:

- the option '-ifa' is used to indicate that the input genome is provided in FASTA format, following by the path to the genome assembly file we want to analyse.
- the option '-s' is used to indicate the bacterial species in the same. This is important for ResFinder to use the antimicrobial panel specific to each bacterial species.
- the option '--acquired' is chosen to detected acquired resistance genes, and
- the option '--point' to scan for AMR chromosomal mutations.
- the option '--outputPath' allows you to specify the name of the output directory where ResFinder files will be stored

Next, we will run ResFinder on the raw sequencing reads of strain 07-02477 (accession number ERR017261).

fastq.gz were previously downloaded directly from the ENA using their FTP links (<https://www.ebi.ac.uk/ena/browser/view/ERR017261>) so they are already available on the working directory.

optional

```
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR017/ERR017261/ERR017261_1.fastq.gz  
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR017/ERR017261/ERR017261_2.fastq.gz
```

```
python -m resfinder -ifq ERR017261_1.fastq.gz ERR017261_2.fastq.gz -s  
"Staphylococcus aureus" --acquired --point --outputPath ERR017261_resfinder
```

In the ResFinder command above note we used the same options as for sample HO50960412 with the exception of '-ifq', used here to specify input fastq file(s).

ResFinder assumes the input to be single-end fastq if only one file is provided after '-ifq', and to be paired-end data if two files are provided instead.

Finally, run the same commands for *S. aureus* sample H65 (Table 1, accession ERR038057):

```
python -m resfinder -ifq ERR038057_1.fastq.gz ERR038057_2.fastq.gz -s  
"Staphylococcus aureus" --acquired --point --outputPath ERR038057_resfinder
```

You can use the commands below to run ResFinder for the three *S. typhi* samples:

```
python -m resfinder -ifq ERR2093245_1.fastq.gz ERR2093245_2.fastq.gz -s  
"Salmonella enterica" --acquired --point --outputPath ERR2093245_resfinder
```

```
python -m resfinder -ifq ERR2093329_1.fastq.gz ERR2093329_2.fastq.gz -s  
"Salmonella enterica" --acquired --point --outputPath ERR2093329_resfinder
```

```
python -m resfinder -ifa CT18.fa -s "Salmonella enterica" --acquired --point  
--outputPath CT18_resfinder
```

6.2.2 Interpreting ResFinder results

Based on the ResFinder commands run in the previous section, you should have obtained a '_resfinder' output directory for each of the samples analysed. Out of the various output files, the following ones are key for interpretation:

- ResFinder_results_table.txt: summary of detected acquired AMR genes by antibiotic class, including BLAST statistics such as percentage of nucleotide identity or percentage of gene length covered.
- PointFinder_table.txt: summary of chromosomal genes scanned for AMR point mutations for the chosen bacterial species.
- PointFinder_results.txt: detected AMR chromosomal point mutations in your sample, and associated phenotypic resistance.
- pheno_table_[species].txt: ResFinder (including PointFinder) WGS-predicted phenotypes for the bacterial species chosen, including the detected genetic determinants supporting such prediction.
- pheno_table.txt: ResFinder (including PointFinder) WGS-predicted phenotypes for all antibiotics included in this database. These results should be interpreted with caution, and the file pheno_table_[species].txt prioritised for reporting.

Out of all these output files, we should focus on pheno_table_[species].txt, as it contains WGS-predicted phenotypes that are specific for the chosen bacterial species.

Based on the ResFinder output files, fill in the tables below to facilitate comparison of WGS-predicted antibiograms between strains.

Summary of ResFinder results for *S. aureus* strains

Antibiotic	HO50960412	07-02477 (ERR017261)	H65 (ERR038057)
Amikacin			
Gentamicin			
Tobramycin			
Kanamycin			
Methicillin			
Penicillin			
Fusidic Acid			
Vancomycin			
Clindamycin			
Erythromycin			
Mupirocin			
Linezolid			
Tetracycline			
Trimethoprim			
Daptomycin			
Rifampicin			
Ciprofloxacin			
Moxifloxacin			
Teicoplanin			

Summary of ResFinder results for *S. typhi* strains

Antibiotic	CT18	BL0006 (ERR2093245)	Pak60168 (ERR2093329)
Ampicillin			
Broad-Spectrum Cephalosporins			
Chloramphenicol			
Ciprofloxacin			
Sulfonamides			
Trimethoprim			
Co-Trimoxazole			
Tetracycline			
Azithromycin			
Colistin			
Meropenem			

6.3 WGS-based prediction of AMR using CARD RGI

6.3.1 Introduction to CARD and RGI

The Comprehensive Antibiotic Resistance Database (CARD) is a very commonly queried database of resistance genes and associated AMR phenotypes.⁸ CARD is a curated resource (<https://card.mcmaster.ca>) providing reference DNA and protein sequences, detection models and bioinformatics tools on the molecular basis of bacterial antimicrobial resistance (AMR). CARD focuses on providing high-quality reference data and molecular sequences within a controlled vocabulary, the Antibiotic Resistance Ontology (ARO), designed by the CARD team.

CARD's Resistance Gene Identifier (RGI) is a bioinformatic tool developed to predict resistomes from genomic and metagenomic data using the CARD database as a source of AMR genes and curated genotype-phenotype relationships. Analyses can be performed via this web portal or via the command line (<https://github.com/arpcard/rgi>), which can be used to automate the analysis of multiple samples in bioinformatic scripts. Briefly, RGI algorithmically predicts AMR genes and mutations from submitted genomes using a combination of open reading frame prediction with Prodigal, sequence alignment with BLAST or DIAMOND, and curated resistance mutations included with the AMR detection model.

6.3.1 CARD RGI commands

Navigate to the 'cp6' directory:

```
cd ~/course/cp6/
```

Activate 'rgi' conda environment:

```
conda activate rgi
```

The raw sequencing reads and genome assemblies used in this practical can be found in the directory `/cps/data/`.

Because the genomes of our strains are available as raw sequencing reads or genome assemblies, we will need to indicate RGI the format of input files. RGI can analyse both paired-end Illumina reads in fastq.gz format with the tool *rgi bwt*, and genome assemblies in FASTA format with the tool *rgi main*.

Execute the commands below to display the arguments and options of each RGI tool:

```
rgi main -h
```

```
rgi bwt -h
```

Next, download a local copy of the latest CARD database:



```
wget https://card.mcmaster.ca/latest/data --no-check-certificate
tar -xvf data ./card.json
rm data
rgi load --card_json card.json --local
rgi database --local --version
```

You can use '*rgi main*' to detect AMR genes from genome assemblies as showed in the commands below:

```
rgi main --input_sequence HO50960412.fa --output_file HO50960412_rgi
--input_type contig --clean --local
```

```
rgi main --input_sequence CT18.fa --output_file CT18_rgi --input_type contig --clean
--local
```

```
rgi main --input_sequence ERR017261.assembly.fa --output_file ERR017261_rgi
--input_type contig --clean --local
```

```
rgi main --input_sequence ERR038057.assembly.fa --output_file ERR038057_rgi
--input_type contig --clean --local
```

```
rgi main --input_sequence ERR2093245.assembly.fa --output_file ERR2093245_rgi
--input_type contig --clean --local
```

```
rgi main --input_sequence ERR2093329.assembly.fa --output_file ERR2093329_rgi
--input_type contig --clean --local
```

In the '*rgi main*' commands above, note the following arguments:

- --input_sequence: file name of the input genome assembly to analyse
- --output_file: name of RGI output file
- --input_type: type of input data, contig (DNA) or protein. Because the default option for this argument is 'contig', '--input_type contig' can be omitted.
- --clean: option to remove temporary files created by RGI
- --local: use local CARD database (the one we just downloaded)

6.3.2 Interpreting CARD RGI results

You can use the 'cut' command below to extract specific fields (i.e. columns) from RGI output files: Cut_Off, ARO, SNPs_in_Best_Hit_ARO, Drug Class, Resistance Mechanism, and AMR Gene Family.

```
cat HO50960412_rgi.txt | cut -d'$\t' -f6,11,13,15,16,17 > HO50960412_rgi.short.txt
```

In RGI output files pay particular attention to the fields 'Drug Class', 'Resistance Mechanism', and 'AMR Gene Family' which you can use to derive to what antibiotic each CARD-identified genetic determinant is conferring resistance to. Based on CARD RGI results, fill in the tables below to facilitate the comparison of genotypic antibiograms.

Summary of CARD RGI results for *S. aureus* strains

Antibiotic	HO5096041 2	07-02477 (ERR0172 61)	H65 (ERR03805 7)
aminoglycoside antibiotic			
carbapenem			
cephalosporin			
fluoroquinolone antibiotic			
glycopeptide antibiotic			
lincosamide antibiotic			
macrolide antibiotic			
monobactam			
nitrofurantoin antibiotic			
oxazolidinone antibiotic			
penam			
penem			
phenicol antibiotic			
rifamycin antibiotic			
streptogramin antibiotic			
sulfonamide antibiotic			
tetracycline antibiotic			

Summary of CARD RGI results for *S. typhi* strains

Antibiotic	CT18	BL0006 (ERR20932 45)	Pak60168 (ERR20933 29)
aminoglycoside antibiotic			
carbapenem			
cephalosporin			
fluoroquinolone antibiotic			
glycopeptide antibiotic			
lincosamide antibiotic			
macrolide antibiotic			
monobactam			
nitrofurantoin antibiotic			
oxazolidinone antibiotic			
penam			

penem			
phenicol antibiotic			
rifamycin antibiotic			
streptogramin antibiotic			
sulfonamide antibiotic			
tetracycline antibiotic			

6.4 WGS-based prediction of AMR using AMRFinderPlus

6.4.1 Introduction to AMRFinderPlus

To enable accurate assessment of AMR gene content, as part of a multi-agency collaboration, the National Center for Biotechnology Information (NCBI) in the US developed a comprehensive AMR gene database, the Bacterial Antimicrobial Resistance Reference Gene Database, and AMRFinder, an AMR gene detection tool.⁹ Recently, NCBI released a new version of AMRFinder, known as AMRFinderPlus that, among several new functionalities, has been expanded to detect point mutations in both protein and nucleotide sequences, and taxon-specific analyses that include, or exclude, certain genes and point mutations for specific taxa. AMRFinderPlus (<https://github.com/ncbi/amr>) is available on as a command-line tool only. In this section we will run AMRFinderPlus on the same strain genomes analysed with ResFinder and CARD RGI in previous sections.

6.4.2 AMRFinderPlus commands

Navigate to the 'cp6':

```
cd ~/course/cp6/
```

The raw sequencing reads and genome assemblies used in this practical can be found in the directory ~/course/cp6/

The only required arguments to run AMRFinderPlus are either -p <protein_fasta> for proteins or -n <nucleotide_fasta> for nucleotides. Use '--help' to see the complete set of options and flags.

```
amrfinder --help
```

Use 'amrfinder -u' to download and prepare database for AMRFinderPlus:

```
amrfinder -u
```

First, a local database of the latest the latest AMR database must be download.

```
mkdir amrfinder_db
```

```
amrfinder_update -d ./amrfinder_db
```

After making sure the latest AMR database is downloaded, you can run amrfinder on genome assemblies, as showed in the command line below:

```
amrfinder -n HO50960412.fa -O Staphylococcus_aureus -o  
HO50960412_amrfinder.txt
```

It should take a couple of minutes for this command to finish.

From the command above, note the following chosen options:

- AMRFinder only supports the processing of input nucleotide sequences in FASTA format (with the `-n/--nucleotide` option), and not the analysis of raw reads in fastq format. This means that raw reads must be *de novo* assembled first.
- The option `-o/--output` allows you to choose the name of the output file.
- One of the strengths of AMRFinder is the option `-O/--organism` which can be used to get organism-specific results. For those organisms which have been curated, using `--organism` will get optimized organism-specific results, and it is therefore recommended. AMRFinderPlus uses the `--organism` for screening for point mutations and to filter out genes that are nearly universal in a group and uninformative.

Use `'amrfinder -l'` to list the organism options supported by AMRFinder:

```
amrfinder -l
```

You will find that `'Staphylococcus_aureus'` and `'Salmonella'` are included in the list of supported taxa.

The list of commands below will run AMRFinder for the remaining samples:

```
amrfinder -n ERR017261.assembly.fa -O Staphylococcus_aureus -o  
ERR017261_amrfinder.txt
```

```
amrfinder -n ERR038057.assembly.fa -O Staphylococcus_aureus -o  
ERR038057_amrfinder.txt
```

```
amrfinder -n CT18.fa -O Salmonella -o CT18_amrfinder.txt
```

```
amrfinder -n ERR2093245.assembly.fa -O Salmonella -o ERR2093245_amrfinder.txt
```

```
amrfinder -n ERR2093329.assembly.fa -O Salmonella -o ERR2093329_amrfinder.txt
```

6.4.3 Interpreting AMRFinderPlus results

The table below includes some of the columns of the AMRFinderPlus output file `HO50960412_amrfinder.txt`.

Gene symbol	Sequence name	Element subtype	Class	Subclass
gyrA_S84L	Staphylococcus aureus quinolone resistant GyrA	POINT	QUINOLONE	QUINOLONE
mecA	PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	AMR	BETA-LACTAM	METHICILLIN
mecR1	beta-lactam sensor/signal transducer MecR1	AMR	BETA-LACTAM	METHICILLIN
tet(38)	tetracycline efflux MFS transporter Tet(38)	AMR	TETRACYCLINE	TETRACYCLINE
23S_C2220T	Staphylococcus aureus linezolid resistant 23S	POINT	OXAZOLIDINONE	LINEZOLID
23S_C2220T	Staphylococcus aureus linezolid resistant 23S	POINT	OXAZOLIDINONE	LINEZOLID
parC_S80F	Staphylococcus aureus quinolone resistant ParC	POINT	QUINOLONE	QUINOLONE
blaI	penicillinase repressor BlaI	AMR	BETA-LACTAM	BETA-LACTAM
blaZ	penicillin-hydrolyzing class A beta-lactamase BlaZ	AMR	BETA-LACTAM	BETA-LACTAM

The column 'Gene symbol' indicates the genetic determinant (either acquired gene or point mutation) associated with phenotypic resistance, the latter indicated in the column 'Subclass'.

Based on AMRFinderPlus output files, fill in the tables below to facilitate comparison of WGS-predicted antibiograms between strains.

Summary of AMRFinderPlus results for *S. aureus* strains

Antibiotic	HO50960412	07-02477 (ERR017261)	H65 (ERR038057)
Amikacin			
Gentamicin			
Tobramycin			
Kanamycin			
Methicillin			
Penicillin			
Fusidic Acid			
Vancomycin			
Clindamycin			
Erythromycin			
Mupirocin			
Linezolid			

Tetracycline			
Trimethoprim			
Daptomycin			
Rifampicin			
Ciprofloxacin			
Moxifloxacin			
Teicoplanin			

Summary of AMRFinderPlus results for *S. typhi* strains

Antibiotic	CT18	BL0006 (ERR2093245)	Pak60168 (ERR2093329)
Ampicillin			
Broad-Spectrum Cephalosporins			
Chloramphenicol			
Ciprofloxacin			
Sulfonamides			
Trimethoprim			
Co-Trimoxazole			
Tetracycline			
Azithromycin			
Colistin			
Meropenem			

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