



## Guidelines

## The role of whole genome sequencing in antimicrobial susceptibility prediction of bacteria: 2025 update from the European Committee on Antimicrobial Susceptibility Testing Subcommittee

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## ABSTRACT

**Scope:** The 2017 European Committee on Antimicrobial Susceptibility Testing (EUCAST) subcommittee report on the role of whole genome sequencing (WGS) in antimicrobial susceptibility testing (AST) concluded that WGS antimicrobial susceptibility prediction (WGS-ASP) was not a sufficiently robust alternative to AST to guide clinical decision making at that stage and that more evidence was required [1]. Since then, the use of WGS, bioinformatic tools, machine learning (ML)/artificial intelligence (AI), databases, and prediction approaches has greatly expanded, along with an increased knowledge of resistance mechanisms and their contribution to antimicrobial susceptibility. In response, a new EUCAST *ad hoc* subcommittee was established in 2024 to review the literature, with the aim of assessing the current potential and limitations of WGS-ASP.

**Methods:** As in the previous report, the subcommittee reviewed the literature on a 'by organism' basis but expanded the list to also include enterococci, *Haemophilus influenzae*, and *Bacteroides fragilis* in addition to those already included in the first version: Enterobacterales, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Clostridioides difficile*, and *Mycobacterium tuberculosis*. Additional sections were included to cover advances in metagenomics, other omics technologies and ML/AI. The full report was compiled and reviewed by all subcommittee members before public consultation in November 2025.

**Conclusions and Recommendations:** Significant progress has been achieved in WGS-ASP, with growing evidence supporting its ability to distinguish wild-type from non-wild-type isolates and, consequently, susceptible from resistant strains, particularly for *M. tuberculosis* and when clinical breakpoints align with the epidemiological cut-off (ECOFF). Despite these advances, important challenges remain before WGS-ASP can be adopted as a clinical decision-making tool. Addressing these gaps will require integrated phenotypic and genotypic surveillance to strengthen the evidence base for complex resistance mechanisms and newer antimicrobial agents, alongside comparative assessments that consider both ECOFF and clinical breakpoints. The analyses will require reference method phenotypic AST and high-quality genomic data. It is critical to ensure that datasets reflect the target populations and encompass the full spectrum of antimicrobial susceptibility, while developing unified interpretation frameworks and harmonized bioinformatics tools to standardize outputs. Robust external quality assessment schemes will be essential for clinical validation, and emerging technologies such as AI and ML offer promising avenues to enhance predictive accuracy. Finally, improvements in cost and turnaround time, coupled with evaluations of setting-specific cost-effectiveness, will be key to enabling practical implementation of WGS-ASP. **Ørjan Samuelsen, Clin Microbiol Infect 2026;32:S3**

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## Scope, context, questions addressed and methods

In 2017, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) subcommittee on the role of whole genome sequencing (WGS) in bacterial antimicrobial susceptibility testing (AST) published a status report on the available evidence for predicting antimicrobial susceptibility using WGS [1]. The report concluded that WGS antimicrobial susceptibility prediction (WGS-ASP) was not a sufficiently robust alternative to AST to guide clinical decision making at that stage and that more evidence was required. Since then, the use of WGS, bioinformatic tools, artificial intelligence (AI), databases and prediction approaches has greatly expanded, along with an increased knowledge on resistance mechanisms and their contribution to antimicrobial susceptibility. In response, a new EUCAST *ad hoc* subcommittee was established in 2024 (<https://www.eucast.org/organization/subcommittees/>

[wgs-and-phenotypic-testing](#)) to review the literature and the progress made since the previous report, with the aim of clarifying and assessing the current potential and limitations of WGS-ASP.

The subcommittee met virtually in September 2024 and agreed on the outline and scope of the report. In a similar manner to 2017, the subcommittee reviewed the literature on a 'by organism' basis but expanded the list of organisms to also include enterococci, *Haemophilus influenzae* and *Bacteroides fragilis* in addition to those already included in the first version: Enterobacterales, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Clostridioides difficile* and *Mycobacterium tuberculosis*. Furthermore, additional sections were included to cover advances in metagenomics, other omics technologies and machine learning (ML)/AI. The whole report was compiled and reviewed by all members of the subcommittee before public consultation at: <https://www.eucast.org/>

**Box 1****Summary of conclusion and recommendations**

1. Expansion of the evidence base: although our understanding of genotype–phenotype associations has grown, further expansion of the evidence base is essential—particularly for complex resistance mechanisms such as mutational over-expression, gene copy number variations, porin expression/modification and efflux pumps. In addition, newer antimicrobial agents (e.g.  $\beta$ -lactam– $\beta$ -lactamase inhibitor combinations, cefiderocol, gepotidacin, zoliflodacin) require more comprehensive data. Ongoing, integrated phenotypic and genotypic AMR surveillance remains critical.
2. Outcome comparators: to advance the applicability of WGS-ASP comparative assessment should use both ECOFF and, if different, clinical breakpoints.
3. Generation of high-quality phenotype–genotype data: phenotypic data should be obtained using reference antimicrobial susceptibility testing (AST) methods (ISO-standard broth microdilution, or with some microorganisms/antibiotics agar dilution). Surrogate AST methods should be avoided. MIC values are preferred over categorical S-I-R data to allow for reinterpretation if breakpoints are revised. Genomic data must meet stringent quality thresholds, tailored to species and sequencing platforms, to ensure reliability.
4. Representative strain collections and metadata: datasets used to evaluate phenotype–genotype relationships should reflect the target population and encompass the full susceptibility spectrum, including wild-type (WT), non–wild-type (NWT), and borderline strains with MICs near clinical breakpoints. Rich contextual metadata is essential for accurate interpretation and to minimize bias.
5. Standardization and advancement of organism-specific interpretive criteria: further development of unified, organism-specific genotype interpretation frameworks is needed—similar to the WHO mutation catalogue for *M. tuberculosis*. Harmonization efforts should be supported by tools that standardize outputs from bioinformatics pipelines to ensure consistency across platforms.
6. Compliance with international quality standards: establishment of robust external quality assessments, proficiency testing, and ring trials are vital for validating WGS-ASP in clinical settings. These measures are also necessary to meet regulatory requirements such as IVDR (EMA) and FDA standards—particularly if the results are aimed to be used in patient management decisions.
7. Leverage AI/ML approaches: the potential of AI and ML models should be further explored for WGS-ASP. For this field to advance, it is essential to train the models on reference data, and to compose strain collections according to the principles described above.
8. Further advancements in technology are required in terms of cost and turn-around-time for implementation of WGS as a primary tool for ASP; setting-specific cost-effectiveness of WGS-ASP should be considered.

AI, artificial intelligence; AMR, antimicrobial resistance; EMA, European Medicines Agency; FDA, Food and Drug Administration; ISO, International Organization for Standardization; IVDR, *In vitro* diagnostic regulatory; ML, machine learning; WGS-ASP, whole genome sequencing antimicrobial susceptibility prediction.

[publications-and-documents/consultations](#) until 9 January 2026. The comments from the public consultation were reviewed, responded to and integrated into the final report if deemed relevant by the subcommittee. The summary of conclusions and recommendations is shown in [Box 1](#).

**The phenotype, genotype and definitions of resistance**

This chapter is complementary to the previous chapter on 'Defining resistance' [1]. One important change is the EUCAST revision of the susceptibility category 'I' from 'intermediate' to 'susceptible, increased exposure', moving from one to two susceptible categories, where the 'I' category reminds the user to ensure that the exposure at the site of infection is sufficient. This also means that there is now discordance between EUCAST and Clinical and Laboratory Standards Institute (CLSI) definitions of S and I [2].

Bacterial AST relies primarily on standardized growth-based technologies such as broth microdilution and disk diffusion. Broth microdilution assessment of MIC is the only International Organization for Standardization (ISO)–certified method and thus the reference method to which alternative methods are calibrated [3,4]. As the ISO certified broth microdilution method is for rapidly growing aerobic bacteria, other methods are used for some bacteria and some agents, such as agar dilution for MIC-testing of fosfomycin, anaerobes, and some other fastidious microorganisms as well as the EUCAST reference protocol for broth microdilution of *M. tuberculosis* [5]. The EUCAST disk diffusion method has been

thoroughly calibrated against reference methodology and is presently the only nonreference, standardized method for AST developed and supported by EUCAST.

Although the term may, at the first glance, seem straightforward, 'antimicrobial resistance' (AMR) needs to be uniquely defined depending on the context.

*Clinical resistance*

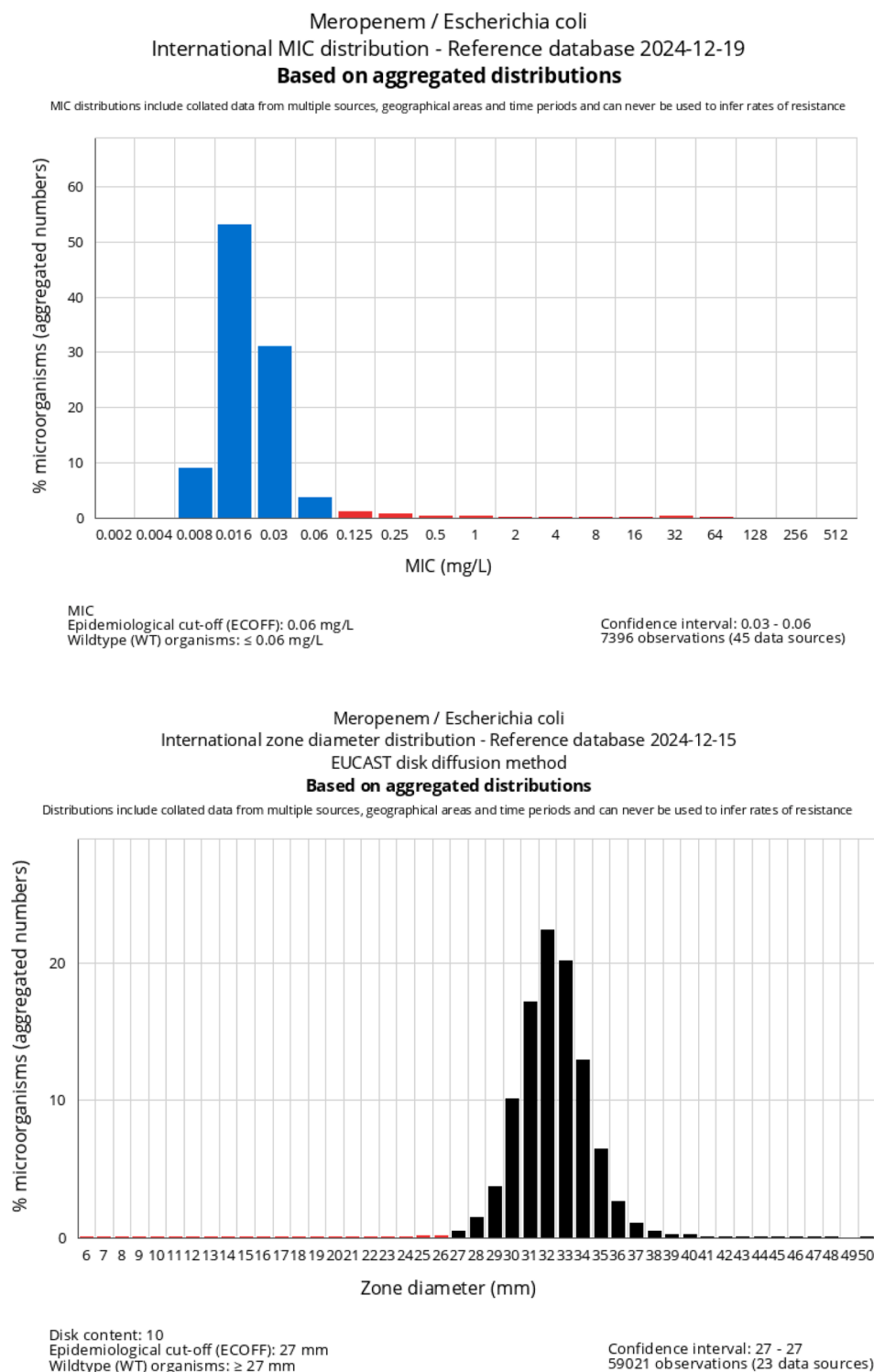
From a clinical perspective AMR occurs when a microorganism fails to be inhibited or killed by the presence of an antimicrobial agent, at any clinically achievable concentration. Clinical resistance is predicted by clinical breakpoints, as published by EUCAST, CLSI, the United States Food and Drug Administration (FDA) and other breakpoint committees or agencies. Clinical breakpoints are used to differentiate isolates for which a given agent there is a high likelihood of therapeutic success from those for which it is unlikely to be effective. They change over time with new knowledge, new indications, new dosing and/or modes of administration and are decided by integrating phenotypic (reference MIC), pharmacokinetic/pharmacodynamic (PK/PD) and clinical outcome studies in humans and/or animal data.

Genotypic data are primarily used to characterize the genetic background to mechanisms of resistance. Importantly, PK/PD data are always related to phenotypic measurements. In cases where the correlation between genotype and phenotype is considered undisputable (e.g. *mecA/mecC* in *S. aureus* predicting almost all  $\beta$ -lactam

resistance and some of the *van*-genes in enterococci predicting resistance to glycopeptides) and in cases where phenotypic testing may fall short, genotypic data may be used to assist clinical interpretation. Indeed, the WHO endorsed a composite reference standard for rifampicin for *M. tuberculosis* whereby a strain is considered resistant if it is phenotypically resistant and/or based on the genotype to overcome the limitations of both approaches [6].

### Phenotypically expressed resistance

From a biological perspective, acquired AMR occurs when the microorganism, through a genetic event, acquires new phenotypically expressed properties that affect the relationship between the antimicrobial agent and the organism in such a way that higher antimicrobial exposure is required to inhibit growth or kill the



**Fig. 1.** Meropenem (top) MIC ( $n = 7396$  MIC-values from 45 data sources) and (bottom) inhibition zone diameter distributions ( $n = 59\,021$  inhibition zones from 23 data sources) in *Escherichia coli*. The ECOFF for the MIC distribution was calculated to 0.06 mg/L (CI 0.03–0.06) and the zone diameter ECOFF to 27 mm (CI 27–27 mm). Blue bars (top) and black bars (bottom) represent wild-type organisms, whereas red bars (both graphs) represent non-wild-type organisms. ECOFF, epidemiological cut-off.

organism. The MIC has traditionally been used to represent the necessary activity of the agent to achieve inhibition of cell growth. It should be highlighted that some relevant mechanisms used by microorganisms to overcome antimicrobial activity that do not determine a modification of the MICs, such as tolerance, persisters and biofilms fall out of the scope of this document. It should also be noted that the MIC is a relative measurement which may vary significantly with changes in test circumstances. When strict adherence to methodology (ISO standard) is achieved, the value is reproducible to the level  $\pm 1$  MIC dilution step in most cases.

Other phenotypic measurements calibrated to the MIC are often used. This is the case for gradient tests and for disk diffusion where inhibition zones are calibrated to correspond to the MIC. Collecting the MIC-values or inhibition zone diameters of many isolates of a species for an agent will result in distributions like those shown in Fig. 1. Often, a wild-type (WT) distribution representing organisms without any acquired resistance genes/mechanisms can be identified and an epidemiological cut-off (ECOFF) value determined. The latter will represent the highest MIC-value for organisms devoid of resistance in a biological sense [7–9].

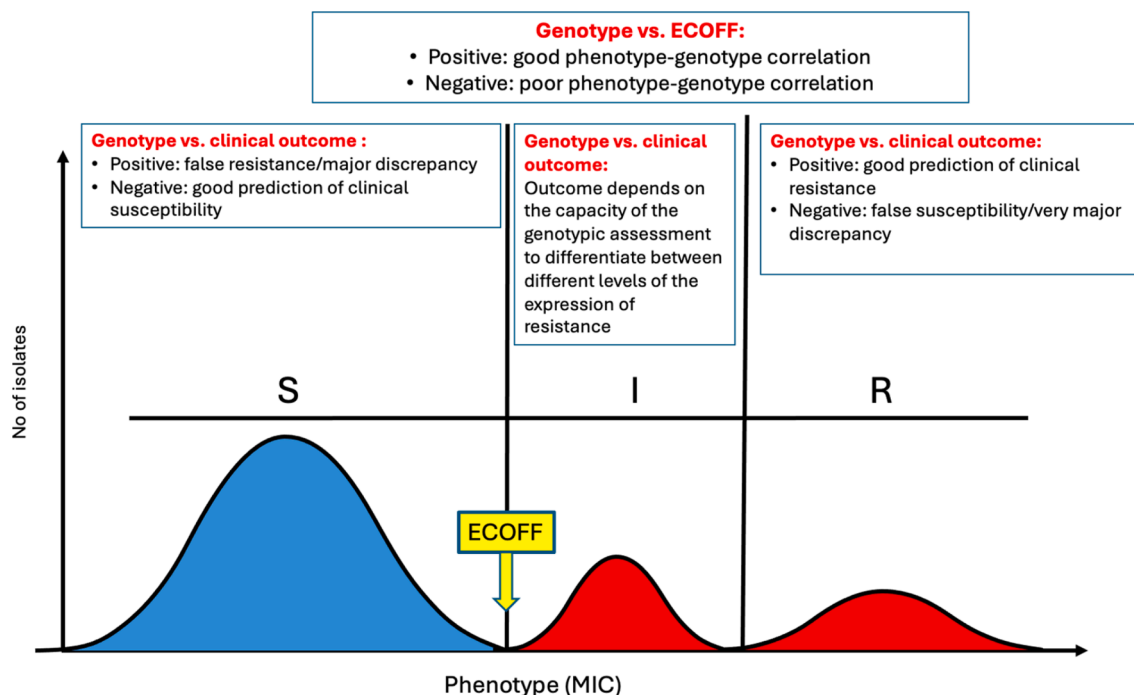
Using a phenotypic measurement, it is possible to identify one main, occasionally the only, population, consisting of organisms entirely devoid of expressed acquired resistance genes or chromosomal mutations. There is evidence that these WT populations, each defined for a single species and a single antimicrobial, are inherent to the species, remain stable over time, and are not affected by the geographical or host origin of the bacterial isolates. One or several secondary populations, representing organisms harbouring identifiable acquired resistance genes or mutations, with higher MIC-values (and smaller zone diameters), appear

outside the WT (non-wild-type [NWT]) because of resistance development.

Since 2003, EUCAST has systematically organized distributions of phenotypic measurements and determined the ECOFFs of these [7]. The organisms belonging to the WT distribution lack phenotypically detectable acquired resistance and thus the ECOFF will distinguish between WT and NWT organisms. However, importantly, WT and NWT are not traditionally defined genotypically and thus extensive WGS data may help to complement these definitions [10]. Also importantly, either distribution can be categorized as clinically susceptible or clinically resistant (Fig. 2).

MIC and zone diameter distributions for many agents and species are freely available through a EUCAST dedicated website (<https://mic.eucast.org>). For each species and agent, EUCAST requires five acceptable and agreeing distributions before an ECOFF is determined using statistical methods and visual inspection [8,9]. The CLSI has a similar but less formal approach to setting ECOFFs, which it abbreviates as ECVs [11].

WT distributions and ECOFFs provide breakpoint committees with a means to avoid setting clinical breakpoints within the WT MIC distributions of target species. A variation in MIC between organisms within the WT is to be expected because of a combination of unavoidable technical variation and any other biological variation between different clones. The natural variation in individual values (MIC or zone diameter measurements) inside the WT distribution does not allow breakpoints to robustly split WT distributions. A breakpoint inside the WT distribution will fail to provide reproducible categorizing of susceptibility and resistance [12]. In addition, if the PK/PD or clinical studies indicate that the achievable exposure of a drug calls for a breakpoint within the WT range, it questions the appropriateness of that species as a target



**Fig. 2.** Simulated illustration of meropenem wild-type (blue) and non-wild-type (red) MIC values for *Pseudomonas aeruginosa* (ECOFF = 2 mg/L). In this example (nonmeningitis infections, clinical breakpoints  $S \leq 2$  mg/L,  $R > 8$  mg/L), the entire wild-type population is treatable with standard exposure (the S-category), and an additional part of the non-wild-type population with increased meropenem exposure (the I category). Isolates with the highest MIC-values are not available for treatment irrespective of exposure (the R-category). From the perspective of mechanisms of resistance, clinical resistance (R) can be typically caused by a single mechanism (e.g. production of a carbapenemase) or by the combination of two low-level resistance mechanisms (e.g. OprD porin inactivation + MexAB-OprM efflux pump overexpression), whereas non-wild-type isolates with low-level resistance mechanisms may still be treatable. However, for other species, agents or infections the scenario may be different, and the wild-type can be categorized I or R instead of S (e.g. carbapenems and *Stenotrophomonas maltophilia*). Moreover, in the same example of *P. aeruginosa* and meropenem, the scenario would be different for meningitis where the resistance breakpoint would be equal to the ECOFF (2 mg/L) and therefore all non-wild-type isolates categorized as resistant. ECOFF, epidemiological cut-off.

for the agent, unless exposure can be improved (i.e. by dose, frequency or mode of administration) [13].

### Resistance genotypes

From a genetic perspective, AMR arises when the microorganism, after an evolutionary event, acquires one or several new functional changes modifying the relationship between the microorganism and the antimicrobial agent. To be associated with AMR, the genetic change will result in the need for increased exposure of the antimicrobial agent to kill or inhibit growth of the microorganism. Such genetic changes are known as ‘resistance determinants’ or ‘resistance genotypes’ and are defined relative to the ancestral genetic background that corresponds to the phenotypically WT population.

### A phenotypic or genotypic definition of WT and NWT organisms

Identifying WT and NWT organisms with genetic or phenotypic methods almost always achieves the same result in most cases. Theoretically, all organisms belonging to the WT should be devoid of phenotypically and genotypically detectable acquired resistance mechanisms and the match between phenotype and genotype should be perfect. However, neither is always the case as exemplified in Fig. 2 and Table 1 [14].

**Table 1**  
Examples of situations that can lead to imperfect phenotype–genotype discrepancies.

| Situation                                                                                                                                                                                                                                        | Examples                                                                                                                                                                                                                                                                             |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| The genotype of phenotypically expressed resistance may not yet have been determined.                                                                                                                                                            | Lack of knowledge of resistance mechanisms to new agents. Introduction of cefiderocol led to identification of mutations in iron transport systems affecting cefiderocol susceptibility. Same applied earlier to <i>qnr</i> genes and fluoroquinolone resistance.                    |
| Genes are not always phenotypically expressed, and gene alteration (mutations, insertions and/or deletions) may be a simple polymorphism without phenotypic effects (e.g. synonymous mutations, or post-translational modification of proteins). | <i>S. aureus</i> with cryptic methicillin resistance. Vancomycin variable enterococci (VVE). Expression of AmpC, efflux pumps and/or OprD in <i>P. aeruginosa</i>                                                                                                                    |
| Phenotypic expression may require the coexistence of two or more genes, including not only resistance but also regulatory genes.                                                                                                                 | Insertion sequence mediated expression of intrinsic genes (e.g. <i>bla<sub>CRA</sub></i> in <i>B. fragilis</i> and <i>bla<sub>OXA-51</sub></i> in <i>A. baumannii</i> ). Presence of a functional <i>ampR</i> required for AmpC expression                                           |
| Overlap between WT and NWT isolates at the upper extreme end of the WT and NWT distributions. In the overlap area, the proportion of NWT organisms decreases rapidly as soon as values are on or below the ECOFF and vice versa.                 | Carbapenemases with low-level activity against carbapenems (e.g. OXA-48-like enzymes).                                                                                                                                                                                               |
| Some phenotypic expressions may require more sensitive systems than those which appear obvious. An alternative molecule may better distinguish between WT and NWT than the target agent.                                                         | Use of pefloxacin to increase the sensitivity of the phenotypic detection of fluoroquinolone resistance in many Gram-negative organisms, including <i>Salmonella enterica</i> . Use of low-concentration benzylpenicillin for the detection of PBP-mutations in <i>H. influenzae</i> |

ECOFF, epidemiological cut-off; NWT, non–wild-type; WT, wild-type.

### Intrinsic or natural resistance and expected resistant phenotypes

Intrinsic resistance i.e. resistance naturally present in the organism, contributing to MICs higher than any clinically achievable concentration, is a theoretical term which has now been abandoned by EUCAST in favour of the term ‘expected resistant phenotype’. This is (in EUCAST) balanced by the term ‘expected susceptible phenotype’ [15]. For a species to have an ‘expected resistant phenotype’, 90% or more isolates irrespective of origin should be considered ‘resistant’ (e.g. *Klebsiella pneumoniae* vs. ampicillin). For a species to have an ‘expected susceptible phenotype’ the WT population should be considered generally susceptible to the agent and a very high proportion (99%) of isolates, irrespective of origin, should be devoid of acquired resistance to the agent (e.g. *Streptococcus pyogenes* vs. benzylpenicillin).

### Antimicrobial resistance development

‘AMR development’ is a frequently used term and mostly refers to a loosely defined ‘general increase in the occurrence of resistant microorganisms’ in an environment, which may be the world at large, a country, a hospital or a single ward. However, theoretically it may refer to more than one phenomenon and the term may be broken down into different parts and should be defined when used:

- The initial occurrence of a previously unknown resistance gene/mutation in any species, of direct or indirect medical interest or of no medical interest.
- The dissemination of a resistance gene to more than one distinctive clone within the species.
- The dissemination of a resistance gene to one or more species.
- An increase in the number of different resistance genes coding for an increasing number of resistance mechanisms.
- An increase in the number of clones in a species which harbour resistance mechanisms to the agent.

Notably, the measurement of AMR frequency, which is required to define expected phenotypes, is always related to some selection criteria; where, when, in whom, why, with which pre-selection? The most severe bias is when there is a preselection of bacteria with related resistance or even unrelated resistance [16].

In summary, the term ‘antimicrobial resistance’ can only be properly understood when the context is known. Resistance genotypes and phenotypes are often, but not always, concordant when considered in the context of WT and NWT. Clinical resistance is entirely related to the predicted outcome of therapy with the agent against the species at a clinically relevant antimicrobial exposure. Isolates displaying resistance in a biological sense, i.e. with resistance genes and mechanisms, may still be considered clinically susceptible, and isolates devoid of acquired resistance genes may be considered clinically resistant.

### Advancements in technologies for WGS-ASP

WGS of bacterial isolates refers to the sequencing of total genomic DNA extracted from a bacterial culture. It can be used to detect the presence of resistance-associated genes or mutations (‘resistance genotyping’), which can in turn be used to predict resistance phenotypes. As highlighted in the 2017 report [1], although WGS is more revealing than conventional molecular approaches, resistome analysis and ASP can be quite challenging.

Thanks to the massive parallelization, short-read sequencing platforms are much more cost-effective than prior sequencing

technologies and, as a result, have been widely adopted in clinical and national reference microbiology laboratories for a variety of purposes [17,18], including outbreak monitoring where WGS-based approaches have become the reference standard because of their superior resolution [19–21]. One of the most remarkable features of these sequencing platforms is their ability to generate highly accurate data, a very valuable trait for resistome analysis purposes, especially for the detection of specific point mutations. Short reads can be directly mapped to reference genes and/or point resistance mutation databases to detect their presence within the bacterial genomes. However, in some cases, an accurate reconstruction of gene order and neighbouring sequences is required to study the functionality or expression of detected resistance genes. In such scenarios, short-read sequencing poses several limitations as resulting reads (50–500 bp) are frequently shorter than the genomic regions of interest and, thus, *de novo* assembly or reference-based mapping of reads is required. *De novo* assembly may fail to resolve these genomic regions when hyper-variable or repetitive segments are present and, therefore, genes associated with AMR may split across several contigs. In addition, within incomplete assemblies, the origin of the resulting fragments is frequently unclear making it difficult to differentiate between plasmid-encoded and chromosomal genes, limiting the application of this approach to investigate transmission pathways of AMR genes. This limitation is particularly significant because resistance genes frequently reside on mobile genetic elements, which are very difficult to reconstruct using this approach [22]. Likewise, another notable shortcoming of *de novo* short-read assemblies is that it frequently fails to determine the exact copy number of multicopy resistance genes because these are often collapsed into a single consensus gene sequence. Finally, as assembler tools prioritize the majority base call, the consensus sequence of a multicopy resistance gene typically reflects the WT nucleotide that challenges the possibility of detecting the resistance mutations present at low frequencies. This can be particularly important in some specific scenarios, such as determining the fraction of 23S rRNA alleles carrying mutations associated with linezolid resistance in enterococci.

On the other hand, the reference-based mapping may also fail to capture the real genetic diversity of the sample, as this approach assumes a predefined genetic arrangement in advance and samples often differ significantly from the reference used.

In contrast to short-read sequencing platforms, long or ultra-long read sequencing platforms (up to 50 kbp) can effectively overcome many of the inherent challenges associated with short-read assembly as long reads significantly enhance the ability to *de novo* assemble complex genomes by providing continuous sequences that span repetitive or structurally challenging regions. Single-molecule real-time and nanopore long-read platforms have historically lagged behind short-read ones in terms of cost-effectiveness and/or error rates [23,24]. However, this scenario is changing, as in recent years, the cost of long-read nanopore-based sequencing has continually decreased, whereas accuracy has been improved [25], although it may still benefit from polishing with short reads, especially when coverage depth is under 100× [26–28] or if a deep learning variant caller is not used for the study of point resistance mutations [29]. Therefore, although considerably more expensive, a common approach is to combine short reads with long-read Nanopore-based sequencing to obtain hybrid assemblies that benefit from the strengths of both technologies. Nonetheless, developments such as proximity scoring with K-mer alignment may offer a promising alternative to polishing in specific contexts such as for determining specific alleles of  $\beta$ -lactamases [30]. In this point, it is important to note that long-read nanopore sequencing offers two major approaches for WGS: one

with a ligation step during library preparation and the other without (rapid), with rapid-based workflows recommended when small plasmid recovery is required [31].

As an alternative, high-fidelity single-molecule real-time sequencing can also be used as a standalone long-read strategy that provides highly accurate long reads; however, its higher cost and lower throughput limit its wide application.

## Quality metrics for WGS-ASP

Establishing stringent quality control (QC) criteria is fundamental to ensuring the reliability of WGS-ASP, as sequencing and metadata quality can significantly impact both sensitivity and specificity of a diagnostic test [32–34]. *In vitro* diagnostic regulatory (IVDR)-based rules need to be followed to use WGS-ASP for diagnostic purposes in the European Union (EU) [35]. In this context, quality refers to the overall integrity, accuracy, and reliability of sequencing data and subsequent analyses [36]. The workflow in the laboratory can be separated into pre- to post-analytical periods, with specific QC considerations at each step [37].

### Preanalytical considerations

These encompass all the steps before DNA sequencing including sample collection, handling and preparation of bacterial cultures, DNA extraction, library preparation, but also metadata documentation. One crucial consideration is the biological diversity of the source. For instance, in cases of urosepsis caused by *E. coli*, the infection has traditionally been considered to be due to a single clone and, therefore, sequencing a single colony could suffice [38]. However, recent developments in our understanding of heteroresistance challenge this assumption [39]. This concept of within-infection diversity has already been recognized for infections involving *Campylobacter* spp., which may require selecting multiple colonies to capture this genetic diversity and avoid missing minority variants that contribute to AMR, which would typically be selected and detected using a phenotypic test [40].

### Analytical and postanalytical considerations

Defining when a sequence is of adequate quality involves setting minimum thresholds for parameters such as read depth and quality, genome size and completeness and indicators of contamination, among others [41]. Coverage that is both adequate in depth and uniform across the genome is key to reducing the risk of missing genes and mutations that mediate AMR. Absence of, or negligible contamination with, DNA of other microorganisms is also a critical quality measurement, as with any microbiological method.

QC thresholds should be set empirically based on observed variation in accuracy of resistance genotyping for individual pathogens [42,43] and ideally recalibrated for the specific WGS workflows in a given laboratory by encompassing sample preparation, library preparation, sequencing technology, bioinformatics workflows, etc. Different pathogens may require tailored QC thresholds because of variations in genome size, complexity and G+C content, which affect sequencing performance (e.g. the GenomeTrakr Project sets different QC thresholds for different enteric pathogens; [44]). Examples of species-specific QC thresholds are listed in Table 2.

**Table 2**  
Quality control threshold guidelines for specific pathogens.

|                                            | GenomeTrackr :<br><i>Salmonella</i> [44] | GenomeTrackr:<br><i>E. coli</i> [44] | KlebNET:<br><i>K. pneumoniae</i> <sup>a</sup> | GPS: <i>S. pneumoniae</i> [45] |
|--------------------------------------------|------------------------------------------|--------------------------------------|-----------------------------------------------|--------------------------------|
| Read quality <sup>b</sup>                  | ≥30                                      | ≥30                                  | –                                             | –                              |
| Read depth <sup>b</sup>                    | ≥30×                                     | ≥40×                                 | ≥40×                                          | ≥20×                           |
| Genome size                                | 4.3–5.2                                  | 4.5–5.9                              | 4.9–6.1                                       | 1.9–2.3                        |
| Contig count <sup>b</sup>                  | ≤300                                     | ≤500                                 | ≤500                                          | ≤500                           |
| G+C content                                | –                                        | –                                    | 56.35–57.98                                   | –                              |
| Proportion of reads matching another genus | –                                        | –                                    | <5%                                           | <2%                            |

<sup>a</sup> <https://bigsdbs.pasteur.fr/klebsiella/genome-quality-check/>.  
<sup>b</sup> For short-read sequencing.

### Platform-specific considerations

Different sequencing platforms generate reads of varying lengths, number, and error profiles, influencing the reliability and interpretation of sequencing results and QC criteria must be adapted accordingly. For critical applications such as single-nucleotide variation detection (e.g. mutations in *gyrA* conferring fluoroquinolone resistance), the accuracy of read-level base calling influences WGS-ASP significantly [46,47] and platforms with high per-base error rates require higher depth thresholds to achieve accuracy comparable with those with low error rates. Nanopore sequencing may fail in reproducing methylation sites and this could affect detection of critical single-nucleotide polymorphisms (SNPs) important for reliable WGS-ASP [46,48]. Coverage and even-ness is important for reliable detection of acquired genes, including plasmid-borne genes, and platforms or library preparation methods that reliably provide even coverage of all DNA regardless of GC content or secondary structures may require less stringent QC in this respect [34,49]. Read length can impact the accuracy of gene copy number estimation [50], resolving insertion sequences (IS) or promoter regions related to expression of resistance genes, and distinguishing whether resistance genes are plasmid-borne or chromosomal. If these factors are known to be important to resistance prediction for a given organism, long-read platforms should be considered along with appropriate thresholds for read length and depth, and assembly completeness.

### The role and availability of proficiency testing for WGS-ASP

Proficiency testing (PT) is key for monitoring the accuracy of laboratory methods, identifying areas for improvement, achieving ISO accreditation and complying with IVDR [35,36]. For WGS-ASP, a method that is not internationally standardized, PT also provides laboratories with an opportunity to benchmark their performance against peers and foster a collaborative environment where best practices are shared, ultimately advancing the field [51].

In a PT for WGS-ASP, test material can be live bacterial cultures, already extracted DNA, or sequence data (FASTQ and/or FASTA files). Depending on the test material, the PT allows assessment of the laboratory capacity for (a) management of live bacterial cultures; (b) DNA extraction; (c) library preparation; (d) sequencing; and/or (e) bioinformatics analyses. Assessment of the participants' proficiency can be conducted using two approaches. One approach consists of evaluating performance against a protocol issued by the PT provider. This approach has the advantage of leading to a high participation rate (whereby participation implies submission of the results to the PT provider). Examples of this approach, limited to PT relevant for clinical laboratories, are the WGS PT offered by the EURGen-RefLabCap project (European Union Service Contract no. SC 20197401) that assessed proficiency of WGS-based resistance profiling of colistin- and/or carbapenem-resistant

Enterobacterales (CRE), *A. baumannii* and *P. aeruginosa* [<https://www.eurgen-reflabcap.eu/eqa>], and the European Union Reference Laboratory for AMR genomic PT [51]. Another important approach is to instruct the participants to use the protocols usually applied in their laboratory, and thereby evaluate their procedure as such. This approach can result in a limited participation rate as participants unsure of the results they generated may simply omit reporting them. However, it has the advantage of forcing participants to undertake root cause analysis to deal with problems that arise during the analysis of the test material. WGS PTs for clinical microbiology laboratories using this approach have been organized, for example, for *S. aureus* by the ESCMID study group on staphylococci and staphylococcal diseases [52], for Enterobacterales and *A. baumannii* in different clinical and public health settings [53,54] and, more recently, for *S. enterica*, *E. coli* and *Campylobacter coli* [55]. As WGS becomes more integral to AMR diagnosis and surveillance, expanding PT programs and benchmarking datasets (and ultimately ISO accreditation), and developing scoring criteria to measure participants' performance will be essential to maintain consistency and accuracy in data interpretation.

### The importance of metadata

Contextual metadata provides information critical for the optimal interpretation of WGS data. These metadata should be reported in standardized, structured ways, e.g. using ontologies such as SNOMED CT (<https://www.snomed.org>), LOINC (<https://loinc.org>) and the Genomic Epidemiology Ontology (<https://genepio.org>). The relevant metadata include details about the specimen, (e.g. collection date, source), the isolate (e.g. analyses methods used, etc.), the patient (e.g. demographics, date and department of current hospitalization, healthcare history, clinical information, etc.) and the hospital (e.g. geographical location, etc.) [56]. Ensuring the quality, completeness, and close linkage of metadata with the resulting WGS data allows for more precise tracking of AMR patterns and the identification of epidemiological trends. Furthermore, metadata can aid in linking genomic variations to phenotypic resistance, improving the predictive power of WGS. The importance of including standardized epidemiological and clinical metadata when sharing pathogen genome data for public health value has been emphasized by the WHO [57, <https://www.who.int/news-room/articles-detail/online-public-consultation-attributes-and-principles-for-genomic-data-sharing-platforms-supporting-surveillance-of-pathogens-with-epidemic-and-pandemic-potential>] and globally applicable metadata standards are under development by the Public Health Alliance for Genomic Epidemiology (PHA4GE) and the International Pathogen Sequencing Network coordinated by the WHO Pandemic Hub.

Examples of standard procedures to report national surveillance metadata can be found in the protocols of the genomic-

based surveys of CRE and carbapenem-resistant *Acinetobacter baumannii* (CRAb) issued by European Centres for Disease Control [58–60] and the One Health surveillance projects established by US health agencies [44]. PHA4GE have also developed a standard for including sequence QC information in contextual metadata [61].

Like genome data, metadata should comply with the FAIR principle for maximizing the utility of WGS in AMR surveillance and research [62].

## Standardization and availability of AMR databases and tools

### AMR databases: a comprehensive summary

The growing importance of genomics as a tool to study the emergence and epidemiology of AMR requires access to searchable, reliable, and foundational data linking genes and their products to resistance. To address this need, databases that provide essential information about AMR genes/mutations, mechanisms, and their genomic contexts are imperative. These databases are crucial resources for microbiologists, epidemiologists, drug discoverers, and healthcare professionals in identifying, tracking, and studying antimicrobial-resistant bacteria.

Several online AMR databases have been developed, each with unique features catering to different research needs [63]. The most widely used include the Comprehensive Antibiotic Resistance Database (CARD) [64], ResFinder [65], MEGARes [66] and National Center for Biotechnology Information (NCBI) AMRFinderPlus [67]. These are actively maintained and curated. Older databases, such as the Antibiotic Resistance Genes Database, the Functional Antibiotic Resistance Metagenomic Element Database, Resfams and the Antibiotic Resistance Gene-ANNOTation are not regularly updated. Each database varies in scope, data sources, user interface, and analytical capabilities.

CARD is one of the broadest and actively maintained AMR databases [64]. It provides detailed information on resistance genes/mutations, proteins, and mechanisms. To qualify for listing on CARD, each gene must be available in GenBank and associated with a peer-reviewed article that confirms a role in resistance. The development of CARD\*Shark, an AI-powered literature search engine that flags the description of new genes in the literature for manual entry into CARD after verification, ensures that CARD is regularly updated [68]. The core of CARD is the Antibiotic Resistance Ontology, a purpose-built ontology model that provides a hierarchical framework for the characterization of resistance genes and associated metadata. CARD includes individual AMR genes and point mutations in antimicrobial targets that confer resistance. One of its key strengths is its Resistance Gene Identifier (RGI) tool, which allows users to analyse DNA sequences for known resistance genes. The RGI settings can be adjusted to restrict the search to known genes (~100% genetic identity) or lowered to flag genes that share variable similarity to known AMR genes, enabling the identification of novel alleles.

The current version of ResFinder is combined with PointFinder and is designed to identify known AMR genes and chromosomal point mutations that confer resistance in genome sequences [65]. ResFinder's simple web interface makes it highly accessible, even to nonexperts. Although, for example, CARD and AMRFinderPlus include a large number of genes naturally occurring in all isolates of a given species, providing intrinsic resistance, ResFinder is manually curated to contain only genes that have been documented to be horizontally transferred. ResFinder allows for user-defined thresholds (10–100%) for searching of similarity to known genes. ResFinder also provides a prediction of phenotypes for selected bacterial species [69]. AMRFinderPlus, developed by

the NCBI, is a tool for identifying AMR genes and point mutations from genomic data [67]. It searches genome assemblies against three NCBI databases (the Reference Gene Catalog, Reference Gene Hierarchy, and Reference HMM Catalog), which are regularly curated by the NCBI Pathogen Detection team with input from experts across US health agencies [67]. One advantage is its ability to detect gene- and protein-based resistance markers. Using the full version of AMRFinderPlus requires local installation, which makes it less accessible to casual users. Of note, initiatives such as AMRColab enable users without bioinformatics skills to use this tool even in low-resource settings [70].

MEGARes is designed primarily for large-scale metagenomic studies, making it highly useful for researchers studying environmental resistomes and complex microbiomes [66]. It uses a hierarchical classification system that helps categorize resistance genes by function and mechanism. MEGARes incorporates data from CARD and ResFinder, AMRFinderPlus and BacMet, which includes resistance to biocides and metals. Unlike other databases, MEGARes does not have a direct web-based interface, requiring users to work with command-line tools. Similarly, for metagenomic datasets, the ARGprofiler tool and the PanRes database consisting of 14 078 unique resistance genes compiled from amongst other ResFinder, ResFinderFG, CARD, MegaRes and AMRFinderPlus have been developed [71].

Other, more narrowly focused databases such as MUSTARD (gut microbiome) (<https://mgps.eu/Mustard/index.php?id=accueil>), the  $\beta$ -lactamase database (<http://bldb.eu/>) and AMR db for *P. aeruginosa* [72] also exist. The WHO mutation catalogue for *M. tuberculosis* is discussed in the relevant section [73].

In general, users should be aware that there is not always overlap between the major databases and reference sequences, and that nomenclature may differ. In addition, the genotype-to-phenotype mappings can vary between databases, and are also variably defined at drug class/specific drug levels.

In addition to these databases, a large-scale resource linking bacterial genomes with antimicrobial susceptibility and extensive metadata across WHO priority pathogens is CABBAGE: the Comprehensive Assessment of Bacterial-Based AMR prediction from Genotypes. This database consolidates information from publications and existing repositories, representing the largest curated AMR genotype–phenotype resource currently available [74].

The ESCMID Study Group on Epidemiological Marker has established a working group to create systematic rules for the organism-specific interpretation of AMR genotypes derived from pathogen WGS data (<https://github.com/AMRverse/AMRrules>). These 'AMRrules' are not an alternative AMR gene database, but aim to facilitate interpretation of outputs from the most widely used databases.

### Tools for AMR detection on WGS data

Numerous bioinformatic tools for AMR mechanism identification have been developed and are variably often coupled to specific AMR reference databases as detailed above. Most are dependent to some extent on one or more of the databases outlined above, but vary in the details of implementation, including their:

- Focus on isolate vs. metagenomic sequence.
- Target organism(s)—e.g. RGI, ResFinder and AMRFinderPlus are species-agnostic, whereas other tools are tailored for a specific species (e.g. *K. pneumoniae* [75], *M. tuberculosis* [76,77], *P. aeruginosa* [72], *S. aureus* [78]).

- (c) Target AMR mechanism(s)—i.e. gene presence/absence vs. mutations, copy number evaluation [79], specific resistance (e.g.  $\beta$ -lactams) vs. all known mechanisms.
- (d) Compute requirements—i.e. laptop/desktop vs. server/cluster vs. cloud/online, speed, resource use and availability of code.
- (e) Input sequence—i.e. short vs. long reads, reads vs. assemblies, protein vs. nucleotide, input file format requirements (fasta, fastq).
- (f) Underlying methods for calling AMR mechanisms (e.g. nucleotide/protein BLAST [Basic Local Alignment Search Tool], mapping  $\pm$  local assembly, k-mer based evaluations, de Bruijn graph-based algorithms).
- (g) Outputs (i.e. determinants found, prediction of phenotype) and how these are formatted and presented.

Comprehensive summaries of different tools and their features are available elsewhere [80,81]. A notable example is abritAMR, an ISO-certified workflow running AMRFinderPlus but with additional curation and reporting features designed to support clinical and public health applications [36].

Benchmarking and comparative evaluations of the performance of some tools, including the more widely used tools, have been published [82–85], but these typically focus on single or discrete species subsets and have a variable prevalence of AMR mechanisms in the sequence data considered. Performance of AMR genotyping tools should assess how well the tool recovers genetic determinants that it seeks to detect (performance of the algorithm), separately from the question of how comprehensive the reference set of genetic determinants is (performance of the database), and subsequently how well these determinants predict phenotypic resistance [10,36].

There is no unified consensus on a benchmarking strategy or availability of a standard benchmarking dataset, although some ideas have been proposed [86,87]. Combining the use of simulated datasets, where the ground truth is clearly known, with real-life datasets, where biological variability may be more accurately reflected, would be helpful. For simulated datasets this would enable accurate characterization of the impacts on performance of aspects such as flanking context of the AMR mechanism, sequencing depth and quality, and presence of closely related alleles, alongside reproducibility [82]. Inter-laboratory comparison studies are also important to assess how different tools are implemented and interpreted by downstream users [54]. To support systematic comparison of AMR genotyping tools, PHA4GE developed the hAMRionization tool (<https://github.com/pha4ge/hAMRionization>), which processes the outputs of 18 different AMR genotypers into a consistent format, facilitating direct comparison of results [88]. This enables users to rapidly identify discrepancies in calls made by the different tools on the same set of data and facilitate comparison to ground truth (or phenotype) where this is available.

## Recent advances in WGS-ASP: Gram-negatives

### *Enterobacterales*

#### Background

The order Enterobacterales is a large and heterogeneous group with variable clinical relevance, intrinsic AMR and potential for development of AMR. Key AMR threats include third-generation cephalosporin/CRE and fluoroquinolone-resistant *Salmonella enterica* serovar Typhi and *Shigella* spp. defined as critical and high priority pathogens, respectively, by the WHO [89]. Our understanding of genotype–phenotype associations among

Enterobacterales has greatly increased since the previous status report [1] through more sequencing, advancement of long-read sequencing, expansion of reference AMR gene databases and development of species-specific tools (e.g. Kleborate for *K. pneumoniae* [75], Typhi Mykrobe for *S. enterica* serovar Typhi [90]). Moreover, ML and statistical modelling methods have enabled prediction of not only WT versus NWT cases and categories according to clinical breakpoints, but increasingly claim to predict MICs within the  $\pm$  one dilution step phenotypic MIC variation [91–95]. However, the increased knowledge has also revealed the complexity of genotype–phenotype associations and identified multiple challenges related to our understanding of the full range and interplay of mechanisms. Further evidence has also been generated for the role of WGS for AMR surveillance and resistance prediction in *Salmonella* spp. [96–100]. Thus, we do not consider there to be major differences in terms of resistance mechanisms that are detected in *Salmonella* spp. compared with other Enterobacterales, therefore *Salmonella* spp. will not be considered separately in this report.

#### Published studies update and current challenges

The genetic diversity of Enterobacterales species challenges the concept of a single set of rules applicable to the whole order. There is increasing evidence that acquired AMR variants confer different phenotypes depending on the species or lineage, e.g. the same *bla*<sub>OXA-48</sub> plasmid resulted in significantly higher ertapenem and piperacillin/tazobactam MICs in *E. coli* compared with *Proteus mirabilis*, whereas meropenem MICs were comparable in both species [101]. This MIC variability is likely caused by differences in intrinsic resistance mechanisms and permeability; thus, assessing different combinations of species/resistance mechanisms is challenging, but indispensable to improve AST prediction in different species. An example of lineage-specific impacts was highlighted through long-read sequencing showing *E. coli* amoxicillin/clavulanate resistance to be impacted by several genotypic features such as promoter variants, gene duplications and copy number, alongside the phylogenetic influence on *bla*<sub>TEM-1</sub> expression [102]. These within-species but lineage-specific differences might also reflect different methylation and different IS/promoter distributions across these lineages, resulting in varying expression, which may be difficult to identify from genomic data. Moreover, variability in the intrinsic resistome also challenges genotype–phenotype associations and interpretation. Ruppé *et al.* [103] showed that prediction of overexpression of AmpC will require more data and that most very major errors (VMEs) were likely associated with overexpression of chromosomal  $\beta$ -lactamase genes (e.g. *bla*<sub>SHV</sub>, *bla*<sub>OXY</sub>). The genetic context and knowledge of which variants are intrinsic or acquired, something which is generally not addressed in AMR gene databases, is also important for interpreting genotype data (e.g. *bla*<sub>OXA-23/-58</sub> in *P. mirabilis* and *E. coli* [104–106]).

Allelic variation leading to phenotypic differences is well known such as mutations in  $\beta$ -lactamases changing the phenotype from a narrow-spectrum  $\beta$ -lactamase to extended-spectrum  $\beta$ -lactamase (ESBL), or ESBL to carbapenemase. However, the allelic variation within resistance gene families is vast and new variants are continuously being discovered. The challenge is that for most allelic variants there is a lack of experimental evidence clearly correlating individual mutations/amino acid changes with phenotype. Consequently, the evidence base for classifying known, but not experimentally validated and new variants is often limited. One way to resolve this is to utilize large collections of matched genotype–phenotype data as done using *K. pneumoniae* isolates ( $n = 3999$ ) with different SHV alleles but no other  $\beta$ -lactamases, which led to the re-classification of several alleles, both from WT

to ESBL and vice versa [107]. Further complicating the role of allelic variation is the observation that synonymous variation and/or uncatalogued variation (i.e. a close but imperfect match to a catalogued reference sequence) is associated with potentially important phenotypic differences [92]. Moreover, within some resistance gene families, e.g. mobile colistin resistance (*mcr*) genes, there are allelic variants that do not confer resistance or cause minor phenotypic changes (e.g. *mcr-9.1*) [108]. However, colistin resistance in diverse species occurs predominantly through spontaneous chromosomal mutation rather than plasmid-mediated resistance [109].

Although specific mutations and truncations within porin genes are increasingly included in AMR databases, we need more knowledge on the mechanisms behind reduced expression of porins and increased expression of efflux pumps. In addition, these mechanisms frequently do not cause resistance alone but contribute to resistance in combination with other mechanisms [110,111].

The advancement of long-read sequencing has shown that gene copy number and gene amplifications can be implicated in the development of resistance to various antimicrobials in particular to  $\beta$ -lactams and  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations (BL/BLI) [92,112–114]. The challenge is that the current tools generally only look for resistance gene/mutation presence/absence and do not evaluate gene copy number. Furthermore, thresholds for the number of gene copies that result in a phenotypically relevant change need to be established.

New treatment options for multidrug-resistant (MDR) Enterobacterales have been introduced such as BL/BLI combinations and cefiderocol. Importantly, the use of these drugs requires an understanding of the underlying resistance mechanisms, which can be provided by diagnostic tests targeting common carbapenemase families or by WGS. With newer antimicrobials unlikely to be included in the first-line testing in routine phenotypic susceptibility testing panels, use of long-read sequencing has shown promise in reducing the time to effective therapy for carbapenem-resistant *K. pneumoniae* compared with standard phenotypic susceptibility testing [115]. However, its cost-benefit must be evaluated further in a real-time clinical setting and compared with alternative approaches (e.g. use of second-line panel testing, direct gene detection etc.).

Introduction of new BLIs such as avibactam, relebactam and vaborbactam has likely led to the selection of novel variants of existing  $\beta$ -lactamases with altered phenotypes, e.g. KPC and VEB variants causing ceftazidime/avibactam resistance [116,117]. Complicating the matter is that some variants are associated with a trade-off, resulting in an altered phenotype against other  $\beta$ -lactams compared with the WT enzyme [118]. This shows the need for continuous genotype–phenotype association studies, which rely on high-quality and quantitative phenotypic data that may not be routinely generated by clinical laboratories. Introduction of new antimicrobials also leads to the emergence of new resistance mechanisms, e.g. reduced expression or mutations in genes involved in iron uptake systems (e.g. *cirA*, *fiu*) is associated with reduced cefiderocol susceptibility [119]. Alterations in PBP genes have generally not been a frequent mechanism of  $\beta$ -lactam resistance in Gram-negatives, but the introduction of aztreonam/avibactam has led to the discovery of PBP3 insertions affecting susceptibility to this agent and also to cefiderocol [120–122]. Genomic approaches may represent an opportunity for novel mechanism discovery by using e.g. genome-wide association studies (GWASs), although typically this requires relatively large datasets where genotype and its effects on phenotypic resistance can be robustly evaluated.

## Summary

Significant advancements have been made on genotype–phenotype predictions in Enterobacterales. However, this has also revealed an increased complexity of resistance mechanisms and we need greater understanding on how these quantitatively impact the phenotype, and clinical outcome. Moreover, with the introduction of new antimicrobials, new mechanisms of resistance will emerge and for some agents, correlation will be hampered by the lack of data from resistant isolates.

## *Pseudomonas aeruginosa*

### Background

*Pseudomonas aeruginosa*, a ubiquitous opportunistic pathogen considered one of the paradigms of AMR, is among the leading causes of hospital-acquired and chronic infections and associated with high morbidity and mortality [123]. Moreover, carbapenem-resistant *P. aeruginosa* was included in the WHO's critical priority pathogen list (2018) reflecting the urgent need for research and development of new antimicrobials [124] and is still considered a high priority in the 2024 updated document [89]. This threat results from the interplay of the extraordinary capacity of *P. aeruginosa* for developing resistance through chromosomal mutations, the increasing prevalence of transferable resistance determinants and the global dissemination of MDR/extensively drug-resistant epidemic lineages, the so-called high-risk clones [125].

The previous review identified the complex repertoire of mutation-driven mechanisms as the main challenge for accurate prediction of susceptibility profiles from WGS, especially when affecting the expression of resistance mechanisms, such as the chromosomal cephalosporinase AmpC or efflux pumps [1].

### Published studies update and current challenges

Although the understanding of the mutational resistome of *P. aeruginosa* and its correlation with the resistance phenotype has incremented to some extent in the last few years [126–128], the assimilation of the quantitative impact and combinatorial effects of resistance mechanisms, as well as the differentiation of mutations from natural polymorphisms, remains challenging. In this sense, Cortes-Lara *et al.* [129] developed and tested a scoring system, based on the analysis of mutation-driven resistance in 40 chromosomal genes and horizontally acquired resistance determinants, for the prediction of ceftazidime, ceftolozane/tazobactam, meropenem, ciprofloxacin and tobramycin susceptibility according to EUCAST clinical breakpoints. Resistance genes/mutations were quantitatively scored from 0 (no effect) to 1 (EUCAST clinical resistance). One hundred fully WT strains were sequenced and analysed to define a catalogue of natural polymorphisms (not involved in resistance) for the 40 chromosomal resistance genes. The scoring system was then evaluated in a multicentre Spanish study, yielding a high accuracy (95–100%) for predicting phenotypic susceptibility. In contrast, the accuracy of the genotypic score to predict resistance was more variable and agent dependent. Prediction of meropenem clinical resistance was particularly low (because of difficulties in differentiating I from R phenotypes), whereas it classified clinical ceftolozane/tazobactam resistance in 100% of cases. The scoring system was further validated in an international cohort, revealing good accuracy in predicting susceptibility (91–100%) and resistance (94–100%) using the same panel of antipseudomonal agents [130]. Madden *et al.* [72] developed a modified, expanded and automatic scoring system linked to the Antimicrobial Resistance Detection and

Prediction software, reaching a genotype–phenotype balanced accuracy of 85% and 81% across 10 clinically relevant antimicrobials when tested against global and validation datasets, respectively. Another developed bioinformatic tool that may help assessing genotype–phenotype correlations is PorinPredict, which predicts defects of the *P. aeruginosa* outer membrane porin OprD, which are strongly associated with reduced carbapenem susceptibility [131]. Moreover, the PaREx tool provides a comprehensive mutational and horizontally-acquired resistome report from *P. aeruginosa* genomes [132]. Beyond the analysis of single resistance mechanisms, the consideration of gene-gene interactions was shown to facilitate the prediction of resistance phenotype, using ciprofloxacin as a model [133]. Moreover, the incorporation of transcriptomics and ML in WGS data analysis resulted in high (>0.8) sensitivity and predictive values for ceftazidime, meropenem, ciprofloxacin and tobramycin resistance [134]. Similarly, Do et al. [135] implemented a pangenome-based ML approach for predicting susceptibility and for discovering novel resistance genes in *P. aeruginosa*. Finally, works have also attempted to analyse the WGS resistance genotype, and its correlation with susceptibility profiles, directly from clinical samples, potentially reducing the diagnostic turnaround time. In this regard, Cortes-Lara et al. [136] validated a hybridization-based capture system to monitor *P. aeruginosa* mutation-driven resistance, and associated susceptibility profiles, in respiratory samples from ICU patients and patients with cystic fibrosis.

## Summary

Recent advances include some improvement in our understanding of genotype–phenotype correlations and the development of scores and tools for the prediction of *P. aeruginosa* susceptibility phenotypes from WGS, which could be eventually correlated to clinical categories, because for the vast majority of antipseudomonal agents, the ECOFF and breakpoints of clinical resistance are identical (<https://mic.eucast.org>). However, challenges imposed by the complex repertoire of mutation-driven resistance mechanisms and the quantitative impact and combinatorial effects of resistance mechanisms persist. Moreover, the introduction into clinical practice of multiple novel antipseudomonal  $\beta$ -lactams is imposing a major increase in the complexity of resistance mechanisms and derived susceptibility profiles, requiring a continuous update of the understanding of genotype–phenotype correlations [137]. Lastly, the availability and accessibility of databases and bioinformatics tools to analyse mutation-driven resistance still needs to be further improved for a broad application.

## *Acinetobacter baumannii*

### Background

*Acinetobacter baumannii* is the most frequently encountered species associated with human infections within the *Acinetobacter baumannii-calcoaceticus* complex [138]. This opportunistic pathogen is frequently implicated in hospital-acquired infections, especially in immunocompromised ICU patients with severe comorbidities. Its capacity to develop carbapenem resistance mostly via acquired carbapenemases contributes to high morbidity and mortality worldwide [139]. Recognized as a critical-priority pathogen by the WHO since 2017 and reaffirmed in 2024, CRAB ranks second globally for mortality related to antimicrobial-resistant infections and is the top pathogen in Southeast Asia [89,139]. Carbapenem resistance prevalence in invasive *A. baumannii* infections often exceeds 40% across Europe, rising to 95% in some regions of Southern and Eastern Europe [140].

### Published studies update and current challenges

Until 2023, few studies had evaluated the performance of WGS-based analyses for predicting antimicrobial susceptibility in *A. baumannii*. One of the earliest studies examined the concordance, using Cohen's Kappa coefficient ( $\kappa$ ), between phenotypic susceptibility determined by disk diffusion and genotypic predictions derived from WGS (via the ResFinder online database) for eight antimicrobial agents in a small collection of 14 *A. baumannii* isolates [141]. Although carbapenem susceptibility was not assessed, the authors reported WGS-based predictive performance according to CLSI breakpoints for ceftazidime (71.4% agreement,  $\kappa = 0.36$ ) and trimethoprim-sulfamethoxazole (92.9% agreement,  $\kappa = 0.81$ ), and a perfect correlation ( $\kappa = 1$ ) was observed between certain *gyrA* and *parC* SNP profiles and ciprofloxacin resistance. However, the small sample size and the low diversity of fluoroquinolone resistance phenotypes is likely to have introduced bias [142].

Another study used the ARESdb platform (Ares Genetics GmbH, Vienna, AT) to predict antimicrobial susceptibility of 72 *A. baumannii* strains, 44% of which were categorized as intermediate or resistant to meropenem, based on agar dilution as the reference method and interpreted using CLSI breakpoints [141]. Overall, the agreement between phenotypic and genomic predictions was 89%, with 8% minor errors, 11% major errors (MEs), and 1% VMEs. Meropenem predictions were the least accurate, with 14% VMEs, 13% MEs, and 14% minor errors. The small sample size and low number of  $\beta$ -lactam-susceptible strains limited this study's robustness [141].

Initially, WGS-ASP relied on rule-based mapping of known AMR genes, requiring pre-existing knowledge. These approaches showed highly variable performances, prompting exploration of ML methods that leverage datasets without prior constraints [143,144]. Among ML strategies, performance can vary considerably. For example, one study found that random-fold partitioning combined with the Kover ML method provided highly accurate AMR predictions in *A. baumannii* [143]. Another study from China utilized a random forest classifier based on 11-mer genomic features [145]. Training was performed on 339 non-duplicate *A. baumannii* isolates collected from 14 teaching hospitals, and testing was conducted on an external set of 120 isolates. When compared with MICs determined by agar dilution for eleven antimicrobial agents including carbapenems and interpreted according to CLSI 2022 guidelines, the overall categorical agreement (CA) and essential agreement (EA) were 97.14% and 94.14%, respectively. Good performance was noted for imipenem (CA = 93.53%, EA = 93.09%), but this was tempered by a VME rate of 8% and a negative predictive value of only 75.25%. Notably, a subset of top-ranked 11-mer features performed as well as the full feature set, demonstrating the potential for streamlined feature selection [145].

An ML-based approach has also been combined with metagenomic next-generation sequencing for rapid ASP in *A. baumannii* [10]. Using a LASSO regression model and a reference database of 1557 *A. baumannii* genomes, key genetic features predictive of resistance (CLSI breakpoints) to ceftazidime ( $n = 31$ ), cefepime ( $n = 24$ ), imipenem ( $n = 20$ ) and ciprofloxacin ( $n = 3$ ) were identified. The model was then applied to 230 retrospective and 50 prospective clinical samples, with results compared with routine culture-based AST performed using Vitek2, which is not a reference method. From metagenomic data, susceptibility results were effectively reported for 80.3% of samples for ciprofloxacin and 94.4% for ceftazidime. When data were available, accuracy exceeded 95% for all four antimicrobials, with positive predictive values above 97% and a perfect (100%) negative predictive value for imipenem. This approach delivered results 44.3 hour faster than conventional culture methods (19.1 hour vs. 63.3 hour) [146].

Finally, a study combined WGS with AMR gene expression profiles and applied a deep neural network model to predict the susceptibility of 497 CRAB isolates to ten antimicrobials [147]. Antimicrobial susceptibility was determined using broth micro-dilution and isolates were categorized using CLSI 2022 breakpoints. Training data were drawn from NCBI GenBank and Biosample Antibigram databases, comprising a total of 1978 genomes. Overall agreement between genotype-based predictions and phenotypes ranged from 91.22% to 98.64%. However, the validation set consisted mostly of OXA-23-producing isolates belonging to ST208 Oxford lineage and included only 21 carbapenem-susceptible strains, limiting the model's generalizability [147].

## Summary

The development of WGS-based technologies for ASP in *A. baumannii* is still in its early stages. Initial approaches rely on detecting known resistance genes, but their performance remains inconsistent, especially for carbapenems. A key limitation is the use of short-read sequencing, which has difficulty resolving the numerous IS identified within or upstream of resistance genes in this species. In addition, there is a lack of well-characterized strain collections that include both carbapenem-susceptible and resistant isolates from various genetic backgrounds.

## *Neisseria gonorrhoeae*

### Background

Antimicrobial-resistant *Neisseria gonorrhoeae* is an urgent global public health concern and listed as 'high priority' by the WHO because of rising prevalence of strains resistant to ceftriaxone, the last remaining approved antimicrobial [89,148]. Diagnosis is commonly syndromic, and treatment is sometimes empiric. Phenotypic AST requires bacterial culture and is performed to varying degrees across countries. Nucleic acid amplification tests are sensitive and specific but do not inform drug susceptibility. Strains resistant or non-susceptible to all approved antimicrobials are rare, for now, raising the possibility that rapid diagnostics that inform drug susceptibility may enable tailored therapy, diversifying the selective pressures driving resistance and prolonging the clinically useful lifespan of ceftriaxone [149–151].

### Published studies update and current challenges

Efforts to develop sequence-based diagnostics rely on understanding the genetic modulators of drug resistance. The optimal case is ciprofloxacin, for which data demonstrate that position 91 in GyrA is reliably predictive, with serine indicating susceptibility, and phenylalanine indicating resistance [152]. Treatment with ciprofloxacin in the context of GyrA 91S has been incorporated into some guidelines [153, <https://sti.guidelines.org.au/sexually-transmissible-infections/gonorrhoea/>].

For other antimicrobials used to treat gonorrhoea, WGS-ASP is more complicated. Moreover, the lack of a global standard for reference MIC-testing of this species remains a challenge when comparing WGS data with MIC-data generated with various MIC surrogate methods. Work has focused on antimicrobials for which *N. gonorrhoeae* are routinely phenotyped in national and international public health surveillance systems. These include the extended spectrum cephalosporin ceftriaxone, the macrolide azithromycin, and tetracycline. Susceptibility to other drugs used for treatment in some parts of the world—e.g. gentamicin and spectinomycin—is not commonly reported, and the paucity of phenotypic data has limited efforts to link genetic variation to MIC or categorical susceptibility testing.

Resistance to ceftriaxone is primarily through mutations in *penA*, which encodes PBP2, the primary target of ceftriaxone [154]. The growing diversity of *penA* alleles resulting in higher range MICs ( $\geq 0.25$  mg/L) [155] challenges the effectiveness of strategies that focus on detection of specific alleles [156], although a study suggests that the PenA A311V mutation may help discriminate [157]. ML approaches have sought to identify highly sensitive and specific markers [158], and linear models suggest MICs determined using the agar dilution method can be predicted by a combination of five amino acids in PenA together with mutations within the regulator of an efflux pump, a porin, and *ponA*, which encodes PBP1 [159].

Multiple pathways can lead to resistance to azithromycin, including ribosomal target site mutations, such as in the 23S rRNA and *rplD* [154,160], and mutations in the *mtrCDE* encoding an efflux pump system and its regulator *mtrR* [154]. Lineages with interspecies mosaicism in the *mtr* locus have been expanding in Europe [161]. Overall, the number of different genetic pathways to resistance and the multicopy 23S rDNA [162] present challenges for sequence-based prediction of azithromycin resistance.

With the introduction of doxycycline postexposure prophylaxis for bacterial sexually transmitted infections (doxy-PEP [163,164]), there is renewed interest in tetracycline resistance. High-level tetracycline resistance (MIC  $>8$  mg/L) is conferred through the plasmid-carried *tet(M)* [165]. The resistance level needed to overcome doxy-PEP is not clear, but it is worth noting that in a study of doxy-PEP use, the rapid increase in the prevalence of tetracycline resistance seems primarily driven by the rise in high-level tetracycline resistant strains [166]. Moreover, in some parts of the world, the prevalence of tetracycline resistance is high [167–169]. As doxy-PEP is more widely adopted, modelling suggests we will see an increase in resistant strains [170], consistent with observations of rapidly increasing prevalence of *tet(M)* in the United States [171].

Two new antimicrobials—gepolidacin and zoliflodacin—have successfully completed phase 3 trials for treatment of gonorrhoea. In gepolidacin's phase 2 trial, resistance developed on treatment, with mutations identified in ParC D86N on a background of GyrA A92T [172]. For zoliflodacin, *in vitro* selection studies identified mutations in GyrB at positions 429, 450, and 467 as contributing to resistance [173]. However, for both drugs, understanding of the diversity of pathways to resistance *in vivo* remains to be described, limiting the applications of WGS-ASP to identifying known resistance determinants.

## Summary

In *N. gonorrhoeae*, the *gyrA* codon 91 genotype can reliably predict the presence and absence of phenotypic resistance to ciprofloxacin but sensitive and specific prediction of phenotype from sequence data is more challenging for other antimicrobials, such as ceftriaxone and azithromycin. The anticipated introduction of two new antimicrobials, gepolidacin and zoliflodacin, underscores the importance of continued surveillance to track the genetic pathways to resistance for each drug.

## *Haemophilus influenzae*

### Background

In 1995, the complete genome sequence of *Haemophilus influenzae* was published, making it the first bacterium to have its full genome sequenced [174]. Despite this milestone, efforts to correlate WGS data with antimicrobial susceptibility phenotypes have not been as extensive as with other pathogens. In most cases, WGS data have been used to identify resistance mechanisms,

particularly those related to  $\beta$ -lactam antimicrobials, and to analyse population structure and serotypes, rather than to establish correlations with clinical categories based on MIC values [175,176].

Reduced susceptibility or resistance to  $\beta$ -lactams in *H. influenzae* is primarily associated with two well-characterized mechanisms [177]. The first one involves transferable  $\beta$ -lactamases, most commonly TEM-1, which confer resistance to ampicillin but not to third-generation cephalosporins. The second mechanism is linked to amino acid substitutions in the transpeptidase domain of PBP3, encoded by the *ftsI* gene. These substitutions can result in varying levels of susceptibility to  $\beta$ -lactams. However, some mutations have no relevant phenotypic effect, and isolates are often categorized as ampicillin-susceptible. Mutations associated with ampicillin resistance are widespread globally, whereas those conferring resistance to cefotaxime, which involve distinct *ftsI* mutations, are more geographically restricted [178].

#### Published studies and current challenges

WGS is gradually being incorporated into surveillance studies of *H. influenzae*. With this approach, a study in Denmark analysing invasive *H. influenzae* isolates reported an overall ampicillin resistance rate of 26.3%. WGS data allowed the classification of isolates into three groups: 10.6% gBLPAR (genetic  $\beta$ -lactamase positive, ampicillin-resistant), 13.6% gBLNAR (genetic  $\beta$ -lactamase negative, ampicillin-resistant) and 2.1% gBLPACR (genetic  $\beta$ -lactamase positive, ampicillin and amoxicillin-clavulanate resistant) [179]. Notably, there was 100% concordance between phenotypic and genotypic  $\beta$ -lactamase production (gBLPAR), as well as for isolates with no resistance genes (gBLNAS, genetic  $\beta$ -lactamase negative, ampicillin-susceptible). However, concordance decreased among gBLNAR isolates, where 13.1% were classified as ampicillin susceptible. Interestingly, gBLPACR isolates also demonstrated 100% concordance between genotype and phenotype. Although resistance genes for other antimicrobial classes (e.g. fluoroquinolones, aminoglycosides, tetracyclines, sulfonamides and macrolides) were also investigated, their phenotypic correlation was not analysed.

A similar study conducted in Norway on invasive *H. influenzae* isolates yielded comparable results [180]. The study confirmed that isolates resistant to aminopenicillins, tetracycline or chloramphenicol possessed relevant resistance markers. Moreover, the absence of substitutions in PBP3 and GyrA/ParC predicted susceptibility to third-generation cephalosporins, meropenem, and fluoroquinolones. However, the detection of mutated PBP3 in *bla*<sub>TEM-1</sub>-negative isolates showed a low positive predictive value for ampicillin resistance (54.4%), indicating that many isolates with PBP3 mutations were still categorized as ampicillin-susceptible based on MIC and clinical breakpoints. For other antimicrobials, the lack of sufficient resistant isolates in the collection limited genotype–phenotype correlation analyses.

Likewise, in a collection of *H. influenzae* from young children with cystic fibrosis including 7 of 23 isolates resistant to ampicillin, 1 of 17 to amoxicillin-clavulanic acid and 8 of 23 to trimethoprim-sulfamethoxazole, the ampicillin-resistance could be explained by  $\beta$ -lactamases or PBP3-changes; however, one case with resistance to amoxicillin-clavulanic acid could not be explained by WGS results. Mutations in the chromosomal dihydropteroate synthase *folP* could explain all cases with trimethoprim-sulfamethoxazole resistance, whereas no *dfr* or *sul* genes were detected [181].

A paper from Switzerland explored the potential effects of mutations in PBP3 on the binding affinity of  $\beta$ -lactam antimicrobials. Group M strains are phenotypically equal to the wild type, whereas amino acid substitutions of group I and group II might affect the  $\beta$ -lactam binding through a common mechanism by disrupting the Asn526-Arg517-Glu324 interaction in PBP3

[182]. A study from Germany investigated the epidemiological significance of PBP3 mutations associated with elevated cefotaxime MIC values (range 0.125–2 mg/L) in *H. influenzae* [183]. Cefotaxime resistance was observed in only 1.1% of isolates. Analysis of the *ftsI* gene revealed that well-characterized PBP3 mutations (e.g. L389F and Y557H) were significantly associated with cefotaxime resistance. However, these mutations were not present in all resistant isolates, suggesting the involvement of additional resistance mechanisms. These findings align with results from a systematic review and meta-analysis, as well as a bacterial GWAS (bGWAS), which demonstrated correlations between PBP3 mutations and resistance phenotypes for both ampicillin and cefotaxime [184].

#### Summary

In conclusion, WGS data can effectively infer phenotypic susceptibility to  $\beta$ -lactams in *H. influenzae* isolates producing TEM-1  $\beta$ -lactamase or with PBP3 mutations. However, this approach may be less reliable in geographic areas in which isolates with certain PBP3 mutations slightly affecting MIC values are widely distributed. For non- $\beta$ -lactam antimicrobials, genotype–phenotype correlations remain underexplored and warrant further studies.

#### *Bacteroides fragilis*

##### Background

Among the anaerobic bacteria, the *Bacteroides fragilis* group comprises the species complex with the highest occurrence of acquired resistance. The most clinically important resistance mechanisms are the metallo- $\beta$ -lactamase encoding gene *cfiA* (conferring resistance to  $\beta$ -lactams including carbapenems), *erm* genes (conferring resistance to clindamycin), and *nim* genes (conferring resistance to metronidazole) [185]. Although EUCAST has no breakpoints for fluoroquinolones, anaerobes can have acquired resistance to this antimicrobial group, which is conferred by the typical mutations in the quinolone-resistance-determining region of *gyrA* as well as drug efflux [186]. In recent years, several global regions have seen increases in resistance levels in the *B. fragilis* group [187,188].

##### Published studies and current challenges

Several attempts have been made to study the correlation of genomic and phenotypic resistance since the late 2010s. Sydenham *et al.* [189] studied six MDR strains of *B. fragilis* sequenced with a combination of short-read and long-read sequencing. In five of the strains with carbapenem resistance the authors could identify *cfiA*, and two of the strains had lower meropenem MICs, consistent with absent IS elements upstream of the *cfiA* genes. The authors identified *nim* genes in four isolates with metronidazole resistance—all of them with complete upstream IS elements. Finally, the four clindamycin resistant strains had *erm* genes. In a later study by Boiten *et al.* [190] the authors sequenced strains with elevated MICs to meropenem, metronidazole and imipenem. Five isolates featured *cfiA*, and three of them with upstream IS elements. The two isolates lacking upstream IS elements had lower carbapenem MICs than the ones with intact elements. Metronidazole resistance was explained by the presence of *nim* genes in three isolates. Again, these results stress that the detection of upstream IS elements is key for correct interpretation of resistance levels [186].

Finally, a study of 174 *B. fragilis* group isolates compared phenotypic methods and WGS. The paper found that high-level carbapenem resistance was often linked to the presence of *cfiA* in combination with upstream IS elements. Contrarily, resistance to

piperacillin-tazobactam seems multifactorial with many relatively poorly understood  $\beta$ -lactamases involved, and potentially also unknown mechanisms. This collection of strains was less suited to study mechanisms of metronidazole resistance [191].

### Summary

In conclusion, the limited existing data suggest that it is possible to predict resistance to metronidazole and clindamycin. For carbapenems high-level resistance is usually related to not only the presence of *cfiA*, but also to upstream IS elements, which could be challenging to predict with short-read sequencing. For other antimicrobials, it remains challenging to define resistance mechanisms correlating with phenotypic resistance.

### Recent advances in WGS-ASP: Gram-positives

#### *Staphylococcus aureus*

##### Background

*S. aureus* exhibits inherent susceptibility to widely used antibacterial drugs. However, core gene alterations or acquisition of AMR genes are occurring and have been linked to drug resistance, creating significant public health problems, with methicillin-resistant *S. aureus* to be considered a priority pathogen by WHO [89]. Although assays such as PBP2a latex testing can rapidly detect methicillin resistance, the ability to predict AMR in *S. aureus* by WGS theoretically might reduce turnaround time for some antimicrobial classes compared with phenotypic methods [192,193] and reveal complex or novel mechanisms for the expression of methicillin and occasionally benzylpenicillin (*blaZ* variants) susceptibility or resistance [194,195]. Resistance genes in *S. aureus* identified by WGS have been pivotal in identifying a wide array of resistance. In our previous report on the role of WGS in AST of *S. aureus*, several studies have been shown to predict AMR from genome sequence data [1].

##### Published studies update and current challenges

Several genomic studies showed that genotype-predicted and phenotypic resistance can agree in up to 95% of cases, especially for methicillin resistance [196–201], whereas penicillin susceptibility may be overestimated when only phenotypic tests and no genomic analysis of *blaZ* is applied [194,195]. Nevertheless, a study by Sabat *et al.* [198] analysed, using WGS, a truly oxacillin-susceptible *mecA*-positive *S. aureus* clonal complex 80 strain that carried SCCmec type IV with a truncated *mecR1* and lacking *mecI*; phenotypic oxacillin susceptibility was linked to a unique plasmid (pGR2A) harbouring a disrupted *bla* system. The *blaR1* sensor gene was nonfunctional because of an IS66 insertion, preventing Bla1 repressor cleavage and *mecA* induction. This genomic study underscored the emergence of adaptive mutations and plasmid-mediated resistance, complicating detection and treatment.

Comparative genomic analyses reported by Di Gregorio *et al.* [192,198], who investigated novel genetic changes in ST8 and ST100 *S. aureus* strains, revealed key genetic changes associated with resistance to oxacillin, vancomycin and daptomycin. With WGS, mutations (SNPs, INDELs [insertions-deletions]) and IS256 insertion identified in genes involved in cell wall metabolism (e.g. *sagB*, *braS*, *stp1*, *yjbH*, *pbp2*) and phosphate regulation (*pitR*, *sarX*), were linked to reduced antimicrobial susceptibility. Notably, in a ST8 strain exhibiting *mec*-independent oxacillin resistance and reduced susceptibility to teicoplanin (MIC 4 mg/L), oxacillin resistance was driven by mutations in *pbp2* (Ala450Asp), *rodA*, *vraT* and *stp1*, affecting peptidoglycan synthesis and  $\beta$ -lactam affinity. Despite hyperproduction of BlaZ  $\beta$ -lactamase, oxacillin resistance

was not attributed to  $\beta$ -lactamase hyperproduction, as clavulanate did not reverse resistance; *pbp2* mutations were evidenced by binding assays and 3D modelling. The gene rearrangement and evolution of stability provided new clues to mechanisms of bacterial adaptation under antimicrobial pressure emphasizing the role of WGS to detect them.

Effective bioinformatics tools are needed to analyse these genetic events and predict their effects on *S. aureus* resistance. Towards this direction, for instance, Mason *et al.* [202] compared three bioinformatics tools (Genefinder, Mykrobe Predictor and Typewriter) for predicting AMR and virulence genes in 1379 *S. aureus* isolates tested by WGS. The methods showed 99.5% concordance in gene detection (Fleiss' Kappa = 0.98), with discrepancies primarily involving virulence genes (e.g. *seu*, *chp*) or resistance genes such as *gacB* and *dfrC*. Phenotypic susceptibility matched genotypic predictions in 98.3% of cases, although disagreements arose for penicillin (false resistance) and gentamicin/ciprofloxacin (false susceptibility). Mykrobe Predictor and Genefinder were faster (<5 min), whereas Typewriter required assembly that needed another 3 hours. This study highlighted the robustness of WGS for resistance prediction but notes trade-offs between speed and specificity, recommending consensus approaches for clinical use. More pipelines for bioinformatics AMR prediction from raw sequencing data have been applied for *S. aureus* resistance detection, particularly ResFinder, Antibiotic Resistance Gene-ANNOTation, PlasmidFinder, PointFinder and CARD, ARIBA [64,70,78,203–205]. For instance, ResFinder 4.0 demonstrated high accuracy in detecting AMR genes and mutations in *S. aureus*, achieving 100% genotype–phenotype concordance for cefoxitin, ciprofloxacin, gentamicin, linezolid and vancomycin making it a reliable tool for surveillance and clinical decision-making [70]. Furthermore, the CARD 2023 includes curated detection models for *S. aureus* resistance genes (e.g. *blaZ*, efflux pumps) and supports ML-driven prediction, enhancing surveillance of AMR in clinical and genomic datasets [64]. Hidden Markov Models combined with ML techniques have been utilized to explore AMR in *S. aureus* by identifying divergent resistance genes and novel determinants, such as tetracycline-inactivating enzymes, which may not be detected by traditional BLAST-based methods. These approaches have been tested on large datasets, including studies involving hundreds of *S. aureus* isolates demonstrating high concordance (>95%) between genomic predictions and phenotypic resistance profiles for multiple antimicrobials [206]. In addition, these models improve forecasts in *S. aureus*, for antimicrobial classes such as glycopeptides, linezolid, daptomycin and  $\beta$ -lactams [202,207].

### Summary

During the last decade several tools and databases have been successfully applied to analyse WGS data on *S. aureus* resistance detection. These tools have improved resistance prediction for several antimicrobials, facilitating effective surveillance and clinical decisions. Predictive capacities may be further improved by ML and transcriptomics research.

#### *Enterococci*

##### Background

The first document [1] lacked a chapter on '*Enterococcus* spp.'. Insofar, we refer here in this chapter to address the key advancements after the 2017 report but also to a point in time before the initial publication (if relevant).

The genus *Enterococcus* includes dozens of known and novel species, many of which were introduced recently, and which were

associated with special ecological niches and as colonizers in specific animal species [208]. From a medical perspective only two species of *Enterococcus* are considered of greater importance, *Enterococcus faecalis* and *Enterococcus faecium*, which we refer to herein. According to recent adaptations in 2024, vancomycin-resistant *E. faecium* remains listed as a 'high-priority pathogen' [89]. Isolates of these two species are regarded as classical nosocomial pathogens associated quite often with higher rates of acquired resistance properties. For most of them, the causal relationship between genotypic changes or acquired resistance genes and a corresponding phenotype is well-described and biologically proven. Problems arise from a number of last resort resistances, where the resistant phenotypes stem from a multifactorial interplay of various mechanisms or complex regulatory circuits involving core and/or accessory genes and/or point mutations [209,210].

Genome-based comparisons revealed the close link between isolates previously categorized as commensal variants of *E. faecium* (clade A2) and which should rather be classified as *Enterococcus lactis* [211,212]. Therefore, it cannot be excluded that in several genome-based studies isolates were included, too, that nowadays would potentially be categorized as *E. lactis*.

Expected resistances exist in the two included species, e.g. against cephalosporins. In *E. faecalis* further insusceptibilities result from a possession of a species-specific gene *lsa(A)* that confers resistance to lincosamides and streptogramins [213]. Acquired resistances to the following drug classes are of importance for treating enterococcal infections: (i) penicillins; (ii) (lipo-)glycopeptides; (iii) aminoglycosides, and last resort agents including linezolid, tigecycline and daptomycin.

#### Published studies and current challenges

Several larger studies based on international collections of strains derived resistant genotypes from WGS data, mainly for glycopeptide resistances [214–217]. This works without problems and an association between presence of the gene clusters and a corresponding phenotype is mainly existent without restrictions. An exception to this are so called isolates of 'vancomycin-variable enterococci' (VVE), which are *vanB*-type or *vanA*-type isolates being phenotypically vancomycin-susceptible, but because of expert opinions should be better reported 'resistant' [218]. Prevalence rates of VVE could reach substantial numbers associated with challenges in the correct genotypic detection of vancomycin resistant enterococci [219]. In the case of VVE, a genomic AMR profile can predict the risk of reversion to phenotypic resistance in phenotypically susceptible isolates [220].

Updates of AMRFinderPlus, ResFinder and other tools also increased prediction quality for AMR profiles from *E. faecalis* and *E. faecium* WGS data [70,221]. When using curated databases and re-testing initially falsely categorized isolates, very accurate genomic predictions were possible for twelve different antimicrobials and using a collection of more than 4000 *E. faecium* genomes [221]. Several years ago, a web-based tool to predict linezolid resistance in enterococci was introduced called LRE-Finder [222]. The tool allows predicting linezolid resistance phenotypes based on 23S rDNA mutations and acquisition of the *optrA*, *cfr* and *poxA* allelic variants.

Colleagues in Australia introduced an ISO-certified workflow designated abritAMR [36]. It is a bioinformatics platform for genomics-based bacterial (including enterococcal) AMR gene detection. The abritAMR platform utilizes AMRFinderPlus. The authors reported that abritAMR displayed 99.9% accuracy, 97.9% sensitivity and 100% specificity (for all targets and bacterial species included).

The authors of one of the aforementioned studies admitted challenges in predicting phenotypes for some last resort antibiotics such as daptomycin (ECOFF according to EUCAST guidance document [[www.eucast.org](http://www.eucast.org), 223]) and tigecycline [221]. Increased MICs against some last resort antibiotics are multifactorial and may result from an interplay of various mutational, regulatory changes and acquired resistances impossible to predict just from genome data [224–228]. The situation is sometimes complicated by the fact that for the newer agents such as lipoglycopeptides clinical breakpoints or ECOFFs are not yet available (e.g. oritavancin), or are only available with a considerable delay (e.g. telavancin, dalbavancin).

#### Summary

Although resistance mechanisms for most antimicrobials and the medically significant species *E. faecalis* and *E. faecium* are well-documented and predictable using genomic data, challenges remain for complex last-resort resistances such as daptomycin and tigecycline, where multifactorial mechanisms complicate accurate predictions. Updates to tools such as AMRFinderPlus, ResFinder, and platforms such as abritAMR have improved AMR prediction accuracy, but limitations persist because of inconsistencies between clinical guidelines (CLSI, EUCAST) and the inability to predict multifactorial resistances.

#### *Streptococcus pneumoniae*

##### Background

*Streptococcus pneumoniae* with macrolide and penicillin resistance are mentioned in the WHO priority document as medium priority [89]. As highlighted in the previous review [1], resistance in pneumococci is largely due to development of mosaic penicillin-binding proteins through transformation, and other target mutations in chromosomal genes [229]. Mechanistic studies have confirmed that the most important mechanisms of resistance to benzylpenicillin and cephalosporins such as cefotaxime is mosaic PBP2x, PBP2b and PBP1a, but there are also other affected genes among those responsible for peptidoglycan synthesis [230,231]. Previous reports also revealed a potential to detect macrolide, tetracycline and fluoroquinolone resistance with WGS-based approaches [232–234], but did not expand this approach to unbiased investigation of clinical isolates.

##### Published studies update and current challenges

Since the previous review, a database for prediction of resistance levels to benzylpenicillin was developed, which is based on amino acid variation in PBP1a, PBP2b, and PBP2x. An investigation of more than 2500 *S. pneumoniae* isolates showed that high-level benzylpenicillin resistance was found in strains with amino acid changes in all three PBPs [235]. However, prediction adapted to the new EUCAST breakpoints ( $S \leq 0.06$  mg/L,  $R > 1$  mg/L) was not specifically investigated. From the publication it seems potentially feasible to define susceptible isolates by having mutations in zero loci, isolates in the I-group by having one mutated locus, and resistant isolates having 2 or 3 mutated loci. Still, there could be overlap between the populations, and the findings should be confirmed in independent studies. The SPN-PBP-AMR prediction module derived from this study was used to investigate strains from a paediatric cohort, while also in the same study using other sources such as CARD, ResFinder and AMRFinderPlus [176]. In total 71 isolates were assessed with phenotypic methods (VITEK2, CLSI guidelines) and although the sensitivity for detecting resistant strains was 100% for ceftriaxone, tetracycline and clindamycin, it was 86% for benzylpenicillin and 78% for clindamycin. It is not

possible to say from the dataset how the data would correlate with broth microdilution using EUCAST criteria.

Furthermore, a study by Metcalf *et al.* [236] investigated 2316 invasive pneumococci that were also subjected to reference broth microdilution. In this study, 104 isolates were not used for  $\beta$ -lactam MIC prediction, because of the mutations not being present in the database, and another seven isolates were excluded because of assembly problems. Some inconsistencies in MIC-prediction were observed in this study, but since then the SPN-PBP-AMR prediction module has been improved with more data. There were some discrepancies in this study with erythromycin, clindamycin, trimethoprim-sulfamethoxazole and tetracyclines, but the overall results suggested reliable prediction of phenotypic resistance to these agents.

Finally, a paper published in late 2024 [237] investigated 517 *S. pneumoniae* isolates with EUCAST disk diffusion and broth microdilution. The distribution of benzylpenicillin susceptibility among the isolates was 79.1% S, 16.8% I and 4.1% R. For other antimicrobial classes, resistance was 21.7% for erythromycin, 15.3% for trimethoprim-sulfamethoxazole, and 22.3% for tetracycline. The study investigated two pipelines—Pathogenwatch and AREScloud. Although CA was above 94% for benzylpenicillin with both pipelines, EA was lower with AREScloud (88.4%)—related to underestimation of benzylpenicillin MICs. For non- $\beta$ -lactams AREScloud had CAs of 95.9% for erythromycin, 93.8% for tetracycline, and 85.5% for trimethoprim-sulfamethoxazole. For Pathogenwatch the CA was lower for the three antimicrobials—88.2% for erythromycin, 81.2% for trimethoprim-sulfamethoxazole and 88.4% for tetracycline. The discrepancies for erythromycin and tetracycline seemed related mostly to suboptimal detection of *erm*(B) and *tet*(M), respectively.

A study employed an integrative approach combining GWASs, resistance reconstruction, and ML to characterize trimethoprim resistance in *S. pneumoniae*, identifying and validating key mutations in *folA* and *sulA* loci. These mutations enabled ML models to accurately predict trimethoprim MICs with an average accuracy of 86.3% within  $\pm 1$  twofold dilution [238].

## Summary

Since the previous review [1], efforts have been made both to improve MIC-prediction of  $\beta$ -lactams and standard S-I-R-prediction of non- $\beta$ -lactams. However, most comparative analyses have not used EUCAST methodology, and moreover there is a need for analysis of how well genomic prediction can separate between S, I, and R with the new EUCAST breakpoints for benzylpenicillin and pneumococci. Potentially, some of these issues could be resolved by reanalysing older datasets that are already available, using the improved pipelines.

## *Clostridioides difficile*

### Background

*Clostridioides difficile* is an anaerobic sporeforming bacterium that can colonize the gut without adverse effects. However, *C. difficile* is globally also one of the leading healthcare-associated infections. Upon dysbiosis of the gut microbiota, often linked to antimicrobial treatment, *C. difficile* may start to produce several toxins, resulting in clinical signs from diarrhoea to potentially fatal pseudomembranous colitis. *C. difficile* can be detected both in humans and animals and zoonotic transmission has been described [239]. Because of reduced effectiveness of antimicrobial treatment for *C. difficile* infection, alternative treatment methods are explored, including faecal transplantation to introduce a stable gut microbiota, and the usage of antibodies against virulence

factors [240,241]. Vancomycin, fidaxomicin and metronidazole are the antimicrobials that presently have received susceptibility breakpoints in the EUCAST system.

### Published studies update and current challenges

The number of studies using WGS to investigate *C. difficile* AMR has increased exponentially over the past decade. Many of these studies report on the acquired AMR-genes, but AST is not frequently performed [242]. Both the PATRIC and NCBI Pathogen detection databases allow for simultaneous reporting of AST and WGS data, but combined these contain less than 800 isolates [243]. The bulk of these isolates are included in the studies described here.

A study by Gargis *et al.* [244] is the largest and most comprehensive study in which WGS and AST is performed on a large collection of 593 isolates from human clinical samples in the United States. The panel of antimicrobials includes vancomycin, metronidazole, moxifloxacin, ceftriaxone, clindamycin and meropenem. Description of the detected AMR-genes is mostly limited to *erm*(B), *erm*(G), *erm*(Q) *cfr*(B), *cfr*(C) and *cfr*(E), linked to susceptibility to clindamycin, as well as *gyrA* and *gyrB* mutations, linked to resistance to moxifloxacin. Although the authors make a compelling breakdown per ribotype, no analysis is performed regarding the concordance of AST and WGS per isolate.

Similarly, smaller studies are described in humans, horses and camelids in China, Australia and the United States, in which both WGS and AST were performed [143,245–247]. Unfortunately, none of these studies have described the correlation and discrepancies between the datasets. Furthermore, the panels of antimicrobials, the methods of AST and the tools for *in silico* detection of genes are not always comparable between the studies.

There is a further need for large scale studies that will perform both AST and WGS through standardized methods, on a wide background of *C. difficile* and apply methods such as GWASs to identify novel AMR-genes and mutations [248].

Fidaxomicin is a relatively novel treatment against *C. difficile* infection. Resistance to fidaxomicin is associated with mutations in the RNase polymerase genes *rpoB* and *rpoC*, which are still rarely reported [249,250].

## Summary

Although some progress has been made in the past decade, the lack of routine AST testing for clinical cases of *C. difficile* results in a very low number of publicly available datasets containing both AST and WGS data. As such, resistance can be predicted mostly for known acquired resistance mechanisms, but the reliability of predictions based on WGS data is limited.

## *Mycobacterium tuberculosis*

### Background

Since 2017, there has been significant progress in the adoption of routine WGS-ASP for the *M. tuberculosis* complex (MTBC) [1]. Specifically, well-resourced settings with a low prevalence of tuberculosis (TB), including the Netherlands, New York State, Sweden, and the United Kingdom, now routinely carry out WGS for most positive MTBC cultures [251–253]. The primary driver is that WGS provides the ultimate molecular resolution for contact tracing [254]. Moreover, in these low-prevalence settings for drug resistance, WGS-ASP has a similar performance to AST in predicting pan-susceptibility to traditional first-line drugs (i.e. isoniazid, rifampicin, ethambutol, and pyrazinamide), resulting in a drastic reduction (67–89%) of AST [251–253]. An external quality assessment (EQA) scheme is available in the EU/European

Economic Area for both WGS uses [255]. Yet, cost and infrastructure requirements for WGS remain high, meaning that routine WGS in high-incidence settings, if available at all, is limited to a small proportion of rifampicin-resistant strains, one of the WHO priority pathogens [89]. Another drawback of WGS is that a positive culture is required, which may take weeks. Instead, targeted NGS assays can be used directly for mostly non-paucibacillary clinical samples and, following their recommendation by WHO, are being adopted in high-incidence TB settings for WGS-ASP for both traditional and some novel drugs, such as bedaquiline [256,257].

#### Published studies update and current challenges

First-generation ASP assays (e.g. line probe or real-time PCR assays) interrogate only the most frequent resistance mutations for a limited number of antimicrobials [257]. By contrast, the second edition of the WHO mutation catalogue, which has become the standard for interpreting WGS-ASP results, provides information for 48,152 mutations and their association with AST results as the reference standard. Specifically, the catalogue distinguishes mutations that can be used as markers for phenotypic resistance from those that are not associated with resistance and mutations for which insufficient evidence exists (Table 3) [73]. Moreover, it provides information about the level of resistance conferred by some agents for which the standard dose can be increased to overcome low-level resistance in some circumstances, such as *inhA* promoter mutations for isoniazid and *gyrA* Ala90Val for moxifloxacin [258,259].

Despite advancements, the implementation of WGS-ASP for MTBC still needs to overcome some challenges:

- (a) Spectrum of resistance mutations. For some drugs, such as the fluoroquinolones and rifampicin (i.e. if essential genes are involved), the spectrum of resistance mutations is limited and can be studied with relative ease (Table 3). For new drugs and in the absence of strong selective pressure

favouring particular mutations in resistance mechanisms involving loss-of-function mutations, the sensitivity of ASP will always be limited, and AST will be required in perpetuity to confidently classify novel mutations in known resistance genes (e.g. for bedaquiline and pretomanid) [73]. In other words, laboratories will always have to supplement the latest edition of the WHO mutation catalogue with resistance mutations that they deem to be relevant to maximize the clinical utility of WGS in their setting.

- (b) Reference AST method only recently endorsed. Unlike for other bacterial pathogens for which standardized reference methods had been in use for many years to minimize the technical variability of AST, no equivalent standard existed for MTBC until EUCAST endorsed a broth microdilution method in 2019 [265]. A refined version of the reference method was used to evaluate two commercial methods for bedaquiline, which is expected to serve as a blueprint for future calibration studies of other anti-TB agents to set sound QC ranges/targets and ECOFFs [5,266–268].
- (c) Clinical trials and breakpoint committees. The design of clinical trials for TB must be improved to generate robust MIC data using the EUCAST reference method that are correlated with clinical and PK/PD evidence for all major MTBC lineages. This would have avoided the situation where WHO has had to recommend the use of the bedaquiline/pretomanid/linezolid regimen for lineage 1 despite very limited clinical evidence compared with lineages 2, 3 and 4 [261]. Given a history of breakpoints without supporting data that turned out to be too high and adversely affected TB treatment (e.g. for rifampicin and moxifloxacin), breakpoint committees should abandon outdated concepts, terminology and, crucially, publish clear justifications for their decisions [6,259,261]. An important issue to consider in this context is the increasing evidence that several key resistance mechanisms confer only modest MIC increases resulting in false-susceptible AST results, which

**Table 3**  
Performance of the second edition of the WHO mutation catalogue for WGS-ASP compared with AST as the reference standard for the entire WHO dataset and using 25% as the allele threshold for calling mutations.

| Drug role  | Drug                      | Resistance genes <sup>a</sup>                                                        | Percentage (95% CI) |                  |                           |
|------------|---------------------------|--------------------------------------------------------------------------------------|---------------------|------------------|---------------------------|
|            |                           |                                                                                      | Sensitivity         | Specificity      | Positive predictive value |
| First-line | Rifampicin                | <i>rpoB</i>                                                                          | 94.4 (94.0–94.7)    | 96.8 (96.6–97.0) | 94.2 (93.8–94.5)          |
|            | Isoniazid                 | <i>inhA</i> and <i>katG</i>                                                          | 92.1 (91.7–92.4)    | 97.9 (97.7–98.0) | 97.0 (96.8–97.2)          |
|            | Ethambutol                | <i>embB</i>                                                                          | 82.1 (81.3–82.9)    | 91.4 (91.1–91.7) | 71.7 (70.9–72.6)          |
|            | Pyrazinamide              | <i>pncA</i>                                                                          | 80.0 (78.8–81.2)    | 97.7 (97.5–98.0) | 90.3 (89.4–91.2)          |
| Group A    | Levofloxacin              | <i>gyrA</i> and <i>gyrB</i>                                                          | 89.2 (88.4–90.0)    | 96.7 (96.4–96.9) | 87.9 (87.0–88.7)          |
|            | Moxifloxacin <sup>b</sup> | <i>gyrA</i> and <i>gyrB</i>                                                          | 90.3 (89.4–91.2)    | 93.2 (92.8–93.5) | 74.0 (72.7–75.2)          |
|            | Bedaquiline               | <i>atpE</i> , <i>pepQ</i> and <i>Rv0678</i> <sup>c</sup>                             | 59.6 (56.5–62.6)    | 98.4 (98.2–98.6) | 75.0 (71.9–77.9)          |
|            | Linezolid                 | <i>rplC</i> and <i>rrl</i>                                                           | 35.0 (30.2–40.1)    | 99.8 (99.7–99.8) | 75.7 (68.6–81.9)          |
| Group B    | Clofazimine               | <i>pepQ</i> and <i>Rv0678</i> <sup>c</sup>                                           | 21.3 (18.2–24.5)    | 98.4 (98.2–98.6) | 38.9 (33.9–44.1)          |
| Group C    | Delamanid                 | <i>ddn</i> , <i>fbiA</i> , <i>fbiB</i> , <i>fbiC</i> , <i>fgd1</i> and <i>Rv2983</i> | 15.1 (10.9–20.1)    | 99.8 (99.7–99.9) | 62.3 (49.0–74.4)          |
|            | Amikacin                  | <i>eis</i> <sup>d</sup> and <i>rrs</i>                                               | 75.4 (73.7–77.1)    | 98.2 (98.0–98.4) | 82.2 (80.6–83.8)          |
|            | Streptomycin              | <i>gid</i> , <i>rpsL</i> and <i>rrs</i>                                              | 80.3 (79.5–81.1)    | 94.0 (93.6–94.4) | 89.8 (89.2–90.4)          |
|            | Ethionamide               | <i>ethA</i> and <i>inhA</i>                                                          | 76.1 (74.9–77.2)    | 85.6 (85.0–86.1) | 63.8 (62.6–65.0)          |

The sensitivity of the catalogue using AST as a reference is likely an underestimate for second-line drugs for which the prevalence of resistance is low because of the low positive predictive value of AST (e.g. for linezolid and delamanid). The low positive predictive value of the catalogue for some drugs (e.g. clofazimine, ethambutol and moxifloxacin) is likely primarily due to mutations that confer only modest MIC increases and/or historical breakpoint that had been too high, resulting in the misclassification of resistant strains as susceptible by AST [73,260]. The performance of WGS-ASP could not be established for cycloserine, imipenem-cilastatin, meropenem, para-aminosalicylic acid and pretomanid as no WHO breakpoints for AST existed when the catalogue was prepared [261–263].

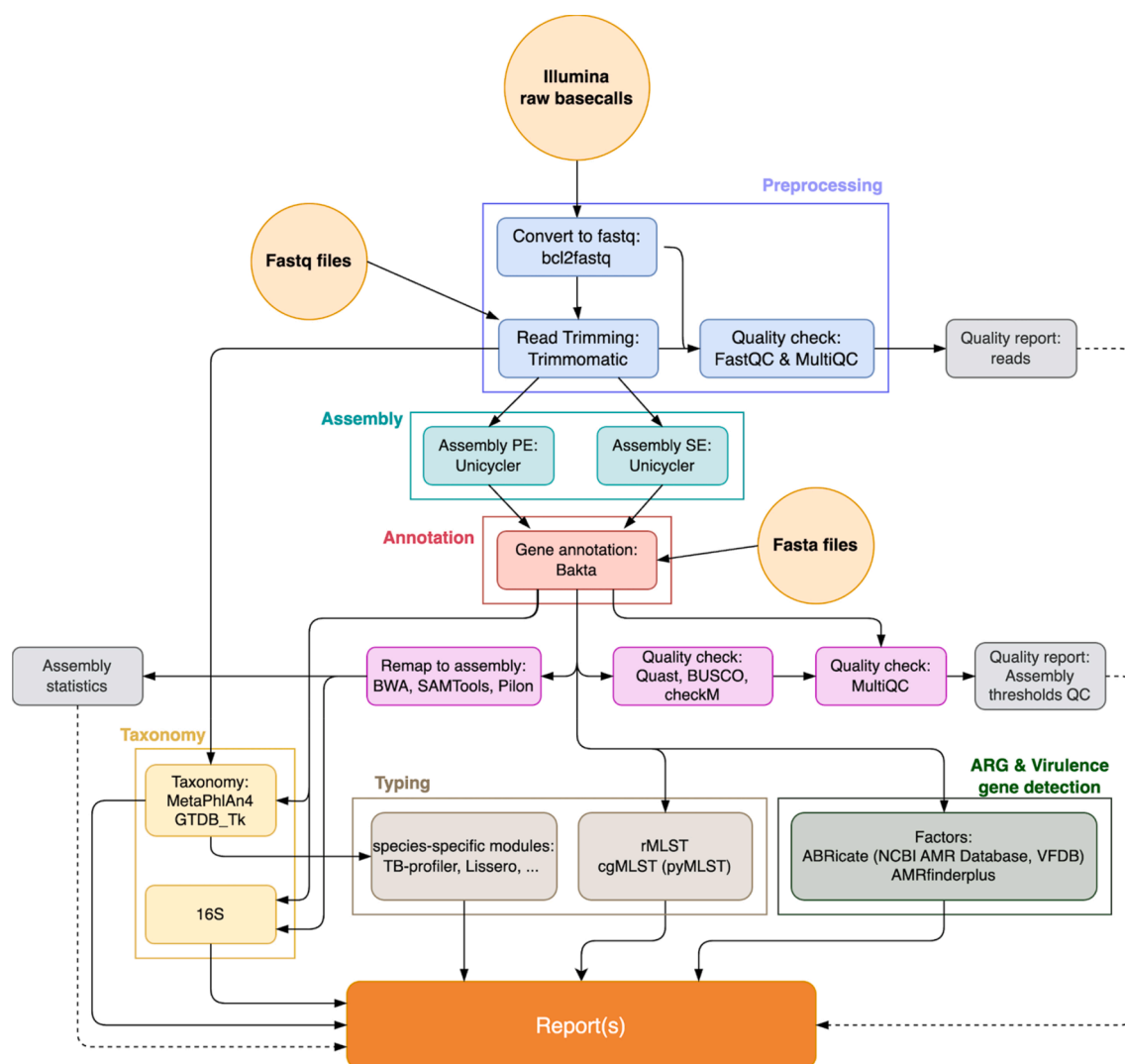
AST, antimicrobial susceptibility testing; WGS-ASP, whole genome sequencing antimicrobial susceptibility prediction.

<sup>a</sup> WHO considered additional genes, but only the genes (or their upstream regions) that were found to contain valid markers of resistance were included in this table.

<sup>b</sup> Is now also considered to be first-line drug by WHO [259].

<sup>c</sup> Epistasis caused by loss-of-function mutations in *mmpL5* must be considered when interpreting *Rv0678* [73,264].

<sup>d</sup> Epistasis caused by loss-of-function mutations in *eis* must be considered when interpreting *eis* promoter mutations [73,265].



**Fig. 3.** IMMense bioinformatic pipeline with ISO-accreditation (diagnostic and molecular surveillance platform used on the Swiss Pathogen Surveillance Platform, [www.spsp.ch](http://www.spsp.ch)). Example of an ISO-accredited bioinformatics pipeline for AMR genotyping (IMMense, used by the Swiss Pathogen Surveillance Platform, [www.spsp.ch](http://www.spsp.ch)). Similar pipelines are implemented in many laboratories, or available in open-source or commercial packages. Numerous interconnected steps are involved, highlighting the challenges inherent in testing, quality controlling, and accrediting such pipelines. AMR, antimicrobial resistance; ISO, International Organization for Standardization.

could be minimized by setting areas of technical uncertainty and endorsing composite reference standards (e.g. for bedaquiline and Rv0678; Table 3) [269].

### Summary

Despite significant progress and the increasing use of routine WGS-ASP, high-quality AST will be required for detecting new resistance mechanisms for MTBC. Coordinated international efforts are needed to improve phenotypic AST as a basis for setting clinical breakpoints and to refine the current mutation catalogue.

### Real-world experience of the application of WGS-ASP in the clinical microbiology lab routine

Since the completion of our last review [1] several additional studies have compared predicted antimicrobial susceptibility based on WGS data against classical culture-based AST methods, including the evaluation of different AI-based algorithms, and now show promise for further evaluating the implementation of WGS-

ASP into practice [36,70,103,143,221,270–274]. For some pathogen-antimicrobial combinations the concordance has reached the FDA acceptance criteria with ME rates <3% and VME rates <1.5% (<https://www.fda.gov/media/88069/download>). However, for specific combinations such as macrolides and tetracycline in streptococci, ciprofloxacin and  $\beta$ -lactams in combination with  $\beta$ -lactamase inhibitors in Enterobacterales, and many antimicrobials in *P. aeruginosa* the discordance between WGS predictions and MIC remains high [273].

Given the improvements in accuracy the use of WGS-ASP is being implemented routinely for surveillance purposes in some settings (e.g. Salmonella AMR surveillance [36,100]) and for guiding initial therapeutic decisions in other settings (e.g. *M. tuberculosis*, [253,275]). However, although WGS clearly shows promise in predicting susceptibility and resistance, there remain significant gaps before this can be used routinely in diagnostics.

Key factors to generate actionable results for antimicrobial stewardship are precision for diagnostics and time from sample collection to result. Beside the requirement on culture-based enrichment, sequencing by synthesis might also add additional

time delays. With recent improvements nanopore-based sequencing might generate data of sufficient quality and in a clinically relevant time-frame [276], issues with methylation and correct base-calling remain [46,48]. In addition, with the current frequency of updates of the software and instrumentation the technology is still not fit for IVDR and thus difficult to implement in settings where IVDR applies. Additional hurdles include ISO accreditation, ring trials, enrolment in commercial EQA programs and easy-to-understand reporting back to clinicians and other healthcare professionals.

#### ISO accreditation, IVDR and EQA

Implementing WGS-ASP in clinical microbiology laboratories requires adherence to international quality standards in many countries, notably ISO accreditation [277]. Although still pending for clinical microbiology, ISO-accredited workflows have been developed for surveillance for example in Australia for routine bacterial pathogens [36] and *M. tuberculosis* [278]. Similarly, the IMMense pipeline has been ISO accredited for short-read data (<https://gitlab.uzh.ch/appliedmicrobiologyresearch/immense>) and is now used for public health, e.g. in the Swiss Pathogen Surveillance platform (Fig. 3).

WGS-based workflows are commonly considered as laboratory-developed tests. The IVDR introduces new regulatory requirements for laboratory-developed tests and diagnostic devices and assays within the EU, directly impacting the implementation of WGS-ASP for routine diagnostic usage. In the United States the regulation is enforced by the FDA. Laboratories must ensure that their WGS-ASP methods comply with IVDR/FDA standards, which may involve comprehensive validation processes and detailed technical documentation of all workflows, including the bioinformatic pipeline. Participation in EQAs is crucial for assessing the accuracy and reproducibility of WGS-ASP results across different settings (see sections above).

#### How to report results

Effective communication of WGS-ASP results is critical for their practical application in patient care. Laboratories must develop standardized reporting formats that translate complex genomic data into clear, actionable information for clinicians [279]. Reports should include detailed findings on detected resistance genes and mutations, but also clinically relevant predictions of impact on antimicrobial efficacy relevant to the pathogen being reported. Providing interpretive comments and recommendations can aid clinicians in selecting appropriate treatments and infection prevention and control teams in the implementation of targeted measures. In addition, reports must comply with legal and ethical guidelines concerning patient data privacy and should be tailored to the needs of stakeholders. An added value could be to also contextualize findings by comparing current data with historical records across time and space, providing insights that were previously unavailable. Clear and concise reporting enhances the utility of WGS-ASP, facilitating timely and informed clinical interventions.

The costs of routine delivery of WGS data to predict antimicrobial susceptibility for patient treatment have not been balanced against potential financial savings across the patient care pathway or the clinical impacts. Previous studies have shown that WGS is economically beneficial for routine surveillance of food-borne pathogens [280] and for preventing hospital outbreaks [281]. Considering that WGS data generated for individual patient care can also be used for surveillance and control of transmission it seems likely that a more widespread use of WGS would be

economically beneficial, but these benefits may translate differently across different settings and should be considered on a case-by-case basis.

If WGS could be made to deliver pathogen identification and predict susceptibility for common pathogens within eight hours after initial culture positivity it may offer enough to impact on measures of patient outcome and use of antimicrobial agents to justify higher costs within the laboratory. It needs to be demonstrated that the implementation of WGS-ASP in the clinic benefits patients, e.g. has the potential to reduce health care-associated infection, or optimize treatments sufficiently to improve outcome and/or stewardship.

Although the cost of WGS will likely limit routine use in low- and middle-income countries in the near future, there are some examples of possible value of the technology in these settings to (a) confirm the genotype causing a certain resistance phenotype that may inform empiric treatment decisions in an outbreak setting [282], (b) detect resistance when phenotypic testing is not available (e.g. to last-line agents such as cefiderocol) and (c) perform direct metagenomics-based diagnostic at locations with no conventional diagnostics [276]. Future efforts should focus on understanding the cost-benefit of using WGS for AMR at the clinical interface in these settings.

#### Inferring antimicrobial susceptibility profiles from metagenomic data

Assessing genotype-to-phenotype relationships directly from clinical samples is even more challenging because of their heterogeneity in terms of bacterial content [283]. In terms of methods, amplicon sequencing (such as 16S profiling) cannot be used as it does not detect AMR genes or genes in which mutations are associated with AMR. Therefore, shotgun (or random) sequencing must be used. Importantly, sequencing clinical samples typically yields a substantial number of human reads, and upstream steps aimed at increasing the proportion of bacterial reads in samples are often necessary [284]. At this point, it is important to consider that the presence of human reads raises ethical concerns, particularly when using online tools or in terms of data sharing, issues that must be handled to ensure compliance with data protection regulations.

Typically, the taxonomic composition of clinical samples can be assessed using specific tools [285] directly from reads or from contigs (obtained via the assembly process). In clinical samples, the number of detected bacteria is variable. Monomicrobial infections constitute the most favourable conditions in that, provided a sufficient depth of sequencing, the whole genome of the causative agent can be reconstructed, providing a situation close to working with genomes. In a study focusing on bone and joint infection samples, a 94.1% concordance between the actual AMR phenotypes and the predictions from metagenomic data [286] was obtained from monomicrobial samples. In other situations, more than one microorganism can be identified (polymicrobial infections) in variable proportions. Hence, the genome of dominant bacteria may be fully reconstructed, whereas the genome of subdominant bacteria may remain partial. In the same study, lower performances (76.5%) were indeed obtained with polymicrobial samples. In a study including sonicated fluid samples from 408 subjects, Yan *et al.* [287] used a commercial bioinformatic pipeline to detect pathogens and predict the antimicrobial susceptibility of *Staphylococcus* spp. when it was found. Metagenomic analysis demonstrated 65.7–85.0% sensitivity and 68.6–100% in predicting staphylococcal resistance by identifying AMR genes associated with methicillin, clindamycin, cotrimoxazole and vancomycin. Sensitivity was affected by the failure to detect AMR genes potentially because of a suboptimal genome

coverage, whereas specificity was affected by the detection of AMR genes carried by surrounding bacteria.

The detection of multiple bacteria indeed raises the challenge of linking AMR genes with their host. Because many AMR genes are embedded in mobile genetic elements, connecting each AMR gene to its host is a challenge bioinformatics has not totally overcome. The Hi-C ligation method can be used during DNA preparation [288], but it has not been applied to clinical samples yet with cost a likely limitation. Thus, linking AMR genes and their host may be subject to the clinical microbiologist's judgment based on existing literature and the known hosts of AMR genes (e.g. the *mecA* gene in staphylococci). In normally sterile samples such as bone and joint samples, the antimicrobial treatment should be active against all the bacteria found. Hence, the presence of AMR genes, irrespective of their host, should be considered when guiding antimicrobial therapy [286] even if variations in the conferred phenotypes could be expected [289]. For instance, if two Enterobacterales species and one ESBL gene (e.g. *bla<sub>CTX-M</sub>*) were found, the production of an ESBL associated with resistance to  $\beta$ -lactams would be considered regardless of the species it belongs to. Samples containing commensals alongside potential pathogens (such as respiratory samples) raise the issue of identifying AMR genes from surrounding commensal bacteria that are actually absent in the pathogen. In a study on 41 respiratory samples, Charalampous *et al.* [290] detected 187 AMR genes among which 24 matched the phenotypic resistance observed in culture, and 14 classified as relevant. However, many of them (98/187) were suspected to originate from commensal bacteria. The choice of the AMR gene database is also important in this regard. As mentioned above, several databases are available, the most popular being ResFinder [70], CARD [64] or the AMRFinderPlus [67] databases which are regularly updated. These databases have been constructed using existing data collected from culturable bacteria, with a bias towards pathogenic bacteria. However, they do not cover the AMR genes found in unculturable bacteria such as microbiota commensals. If AMR genes from these bacteria need to be detected, then specific databases such as ResFinderFG [291] or PanRes [71] could be used to complement conventional databases. However, the spectrum of activity of these AMR genes is not yet known, which limits the clinical interpretation of their potential presence.

Genome coverage is also crucial when attempting to infer genotype-to-phenotype relationships from metagenomic data, with the risk of missing AMR genes if the entire genome has not been sequenced. There is no consensus on the coverage threshold below which some AMR genes could be missed. d'Humières *et al.* [292] observed that a correct phenotype could be assigned when the genome had been reconstructed above 80%, but the number of observations was too low to draw firm conclusions. Missing AMR genes can have sound consequences in that the bacterium may be deemed susceptible to an antimicrobial although actually resistant. Hence, assessing the genome coverage (using tools such as CheckM2 [293] or MetaQUAST [294] of the bacteria found in metagenomes should be performed before inferring antimicrobials susceptibility.

Nonetheless, the sensitivity for detecting AMR genes in clinical samples can be improved by using capture methods [295,296] or amplification [297] before shotgun sequencing, increasing by  $10^3$  [296] to  $10^4$  [297,296] fold the relative abundance of AMR genes in samples.

Besides AMR genes, genes in which mutations can lead to AMR can also be searched for in metagenomic data, such as topoisomerase-encoding genes (*gyrA*, *gyrB*, *parC*, *parE*) associated with resistance to fluoroquinolones [286].

## The role of artificial intelligence (AI) and machine learning (ML) in WGS-ASP

AI is rapidly emerging as a transformative technology, enabling precise, efficient, and scalable solutions to address AMR using WGS data [298]. Key factors for the success of ML-based solutions include (a) the increasingly available well-curated datasets following the FAIR criteria (findable, accessible, interoperable, and reusable) and (b) the affordable computational power required to analyse the data [299].

### Functional ML readouts

ML enables diverse functional readouts for understanding and predicting AMR [300] by identifying patterns in complex genomic datasets [301]. For this task, a range of diverse ML models are commonly used, e.g. random forests (RFs), convolutional neural networks (CNNs), support vector machines and Light Gradient Boosting Machine (LightGBM). These algorithms share specific advantages and disadvantages as depicted in Table 4.

One major application of WGS-ASP is identifying the presence or absence of resistance genes within genomes, which is vital for understanding the genetic basis of resistance. This is achieved through database-driven annotation tools, often paired with curated datasets such as the Bacterial and Viral Bioinformatics Resource Center [306]. ML models further enhance this process by linking gene presence to specific resistance phenotypes at the categorical level.

Another essential readout of WGS-ASP involves identifying SNPs that influence resistance via changes in gene expression or enzymatic activity [307]. Methods such as bGWAS tools, such as Scoary and Pyseer, map SNPs (and genes and k-mers) associated with resistance phenotypes [308,309]. These insights are further validated using integrative workflows that combine bGWAS with whole-genome transformation experiments [310]. In addition, ML models such as LightGBM and CNNs are trained on mutation datasets to predict phenotypic outcomes. For instance, mutations in the *folA* and *sulA* loci have been shown to contribute to trimethoprim resistance in *S. pneumoniae* [238].

A third significant application involves predicting the effects of SNPs on the binding affinity of antimicrobials to their targets [311]. Molecular docking simulations are frequently employed to analyse protein-ligand interactions and assess how specific mutations alter binding. Complementing this approach, methods such as Chaos Game Representation encode genomic sequences to enable high-throughput SNP impact predictions [312]. LightGBM and SNP-based digital MIC prediction tools further enhance the accuracy of these predictions. A compelling example is the *folA* Ile100Leu mutation, which reduces the binding affinity of trimethoprim, providing a mechanistic understanding of resistance [234].

### Challenges and considerations

- (a) Data quality and diversity. ML models depend on high-quality, diverse datasets. However, current AMR genomic resources often exhibit biases in geographic sampling, pathogen representation, quality of the data, and phenotype diversity based on reference methodologies [93]. Expanding these datasets through global collaborations and standardized frameworks is critical as well as to include clinical outcome data.

**Table 4**  
Overview on various ML algorithms used for genotype–phenotype prediction.

| ML model                              | Advantages                                                                                                                                                                           | Disadvantages                                                                                                                                                                    |
|---------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Random Forests [302]                  | Handles high-dimensional data effectively, robust to overfitting due to ensemble averaging. Provides feature importance scores for interpretability.                                 | Computationally intensive with large datasets and may struggle with interpretability for complex decisions. Can be less accurate for datasets with high-dimensional sparse data. |
| Convolutional Neural Networks [303]   | Excels at detecting complex, nonlinear patterns in data. Automated feature extraction reduces reliance on domain-specific knowledge.                                                 | Requires large, labelled datasets and significant computational resources for training. Acts as a ‘black box’, offering limited interpretability.                                |
| Support Vector Machines [302,304]     | Effective in high-dimensional spaces, especially with a clear margin of separation between classes. Works well with smaller datasets and provides flexibility with kernel functions. | Not well suited for very large datasets because of high computational cost. Sensitive to parameter tuning and kernel selection.                                                  |
| Light Gradient Boosting Machine [305] | Highly efficient and scalable for large datasets, with faster training compared with other boosting methods. Handles missing data and provides feature importance.                   | Prone to overfitting if not properly tuned, particularly with small or noisy datasets. Less effective on unstructured data, such as raw text or images, compared with CNNs.      |

CNN, convolutional neural network; ML, machine learning.

- (b) Retrospective datasets. Most of the published WGS-ASP in the ML space is retrospective and there is a clear lack of prospective validation studies. Randomized controlled trials showcasing the added value and feasibility of ML-based algorithms in routine diagnostics are critical.
- (c) Interpretability and validation. Many ML models operate as ‘black boxes’, complicating their clinical adoption. Efforts are needed to develop interpretable models using explainable AI techniques and to validate predictions with independent datasets [93,302].
- (d) Standardization and interoperability. Variability in sequencing platforms, annotation pipelines, and QC criteria hinders reproducibility. Harmonizing methodologies and adopting FAIR principles can ensure consistent performance [93].
- (e) Regulatory concerns. The integration of ML into clinical workflows must address regulatory requirements (e.g. ISO, IVDR, FDA) and ensure data privacy. Following each update of any algorithm, the validation would need to be repeated, which is commonly the case in base-calling algorithms of e.g. Nanopore sequencing [313]. In addition, steps to mitigate algorithmic bias are necessary for equitable applications [35]. This may result in a dependency on large companies being able to provide IVDR-approved products.

Examples of genotype-to-phenotype predictions

There are several examples illustrating the transformative potential of ML to bridge the genotype-to-phenotype gap in AMR research, enabling precise prediction of resistance profiles, uncovering novel insights, and supporting clinical decision-making. ML tools integrated with curated databases, such as CARD, have advanced resistome prediction by systematically associating resistance genes and mutations with antimicrobial classes [64]. CARD’s enhancements, including expanded curation of  $\beta$ -lactamase genes and new ontological frameworks, provide a robust foundation for training ML models.

ML models, such as RFs and CNNs, have achieved exceptional performance in predicting AMR phenotypes. These models often outperform traditional methods by identifying complex, nonlinear relationships between genomic features and resistance phenotypes. For example, RFs and CNNs have demonstrated area under the curve values of up to 0.96 in predicting resistance to antimicrobials such as ciprofloxacin, cefotaxime, ceftazidime, and gentamicin for various pathogens [93]. A notable advantage is their ability to integrate diverse genomic inputs, such as resistance

genes, mutations, and epistatic interactions, to accurately predict resistance.

In *S. pneumoniae*, the integration of bGWAS with ML models has enabled precise identification of resistance mechanisms. These SNP-based ML models have successfully predicted trimethoprim resistance with a  $\pm 1$  two-fold dilution accuracy of 86.3%, highlighting the synergy of statistical and ML approaches in uncovering genotype–phenotype relationships [238].

In *M. tuberculosis*, advancements in resistance prediction have expanded to include phylogeny-aware ML pipelines. For example, the TB-ML framework employs Dockerized ML models for standardized AMR prediction, ensuring reproducibility and interoperability across datasets [64]. Similarly, a novel phylogeny-related parallelism score has been introduced to enhance feature selection by accounting for population structure, leading to improved model performance and identification of biologically relevant SNPs [314]. These pipelines have uncovered known resistance markers, such as mutations in *gyrA* for fluoroquinolone resistance, while also identifying novel candidate mutations [314].

Future trends and conclusion

Future trends in AI and ML for AMR include real-time AMR prediction from WGS, interpretable hybrid models integrating drug properties, AI-driven automated surveillance systems, and personalized antimicrobial therapies. However, regulatory constraints such as strict IVDR limit clinical integration, highlighting the need for multidisciplinary collaboration, global data sharing, and rigorous validation to fully realize AI’s potential in combating AMR.

Role of other omics in WGS-ASP

Although it is relatively straightforward to predict AMR after the identification of the presence of resistance-conferring genes that encode e.g. enzymes that inactivate antimicrobials, it is more complex to predict resistance phenotypes involving the activation of intrinsic resistance-conferring genes [315]. In cases where resistance is due to e.g. overexpression of an efflux pump or the activation of an intrinsic  $\beta$ -lactamase, accurate prediction of the resistance phenotype is more challenging. Many intrinsic resistance mechanisms are regulated by complicated intricate pathways with several players involved in their activation or repression. Inactivation of a broad range of different genes can drive resistance and their inactivation can occur through various mutations, so that predicting the functional outcome of a particular sequence variation in a regulatory gene is difficult. To address this, some have

proposed that measuring the expression level or the functional activity of resistance-related genes could provide a more direct, robust and clinically relevant way to predict resistance phenotypes. This can be achieved either by the application of transcriptomics and qRT-PCR to quantify expression of resistance-conferring genes or the use of mass spectrometry-based detection of their functional activity [316–320].

In addition to genomic mutations in key regulators, the genetic environment of resistance-conferring genes has been shown to impact their expression [321]. Expression levels of resistance-conferring genes can vary significantly across different genomic backgrounds and incorporating transcriptomic information is likely to provide a more accurate prediction of resistance phenotypes than relying solely on identifying these genes through WGS [134,322].

In addition to revealing the presence and expression levels of resistance-conferring genes, profiling cell-wide gene expression levels through other omics approaches has emerged as a new and powerful tool for predicting AMR [323]. Exposure to antimicrobials typically triggers a stress response in susceptible bacteria. This includes activation of pathways that help protect the cell, repair damage, and enable survival. Although a general stress response occurs across many antimicrobials, this response is also specific, varying according to the class of antimicrobial and its target (e.g. cell wall synthesis, protein synthesis, DNA replication) within the bacterial cell. Researchers have exploited the distinct stress responses elicited by different antimicrobials as a strategy to help identify the targets of new antimicrobials with unknown modes of action [324].

On the other hand, the predictable stress responses can also aid in medical microbiology diagnostics. A common method to access antimicrobial specific stress responses is through gene expression profiling. When bacteria are exposed to an antimicrobial with a known target, they exhibit a characteristic transcriptional response, upregulating genes related to the cellular stress pathway engaged by that target. By measuring changes in gene expression using RNA sequencing, the resulting ‘transcriptional fingerprints’ or profiles can provide insights into the isolate’s susceptibility to the antimicrobials [134,322,323,325].

Alongside transcriptional profiling, proteomic profiling offers valuable insights into bacterial responses specific to different types of antimicrobials. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS) enables analysis of the whole cell proteome, allowing AMR to be assessed by detecting proteomic changes after drug treatment in different microbial species and antimicrobials. Of note, as opposed to predicting resistance phenotypes from bacterial genomes and transcriptomes, this approach can provide results within a few hours [326–329]. The results of some earlier studies that aimed to derive a resistance prediction from standard MALDI-ToF MS spectra [330] do not withstand closer inspection as the discriminatory power was found to be insufficient for reliable sub-differentiation [331]. However, measuring more extensive protein spectra with higher-resolution methods as well as the use of AI approaches seems to improve the detection possibilities of AMR markers [332,333].

Previous studies on the resistome of bacterial pathogens indicate that acquisition of intrinsic resistance requires the concerted action of many elements, several of which are integral to bacterial metabolism [334,335]. Just as characteristic transcriptional or translational changes can be detected in bacterial pathogens upon antimicrobial treatment, unique bimolecular fingerprints—including proteins, DNA, lipids, carbohydrates, and metabolites—triggered by specific antimicrobials in resistant versus susceptible isolates can reveal bacterial susceptibility [336,337]. Raman technology-based methods have been used for strain typing [338] and might also emerge as a

valuable, label-free, and non-invasive approach for rapid AST [339–343]. Like recording of MALDI-ToF MS spectra, Raman-based ASP offers a shorter turnaround time than culture-based or WGS-based methods, potentially enabling earlier clinical decisions for treating microbial infections.

Unlike culture-based AST, and the genome-based identification of genetic determinants of AMR, both of which are ‘one-size-fits-all’ approaches, profiling specific bacterial responses with omics technologies requires careful, case-by-case evaluation tailored to different bacterial species or antimicrobials, making these methods less universally applicable [337,344]. In this context, advances in ML promise to improve the predictive power of omics results [345–347], by enabling computational models trained on data from known antimicrobials to predict susceptibility based on observed stress response profiles. These models can also integrate data from multiple omics approaches, potentially increasing diagnostic accuracy and diagnostic speed when using spectroscopic techniques.

## Conclusions and recommendations

The 2017 EUCAST report on WGS as a tool to infer antimicrobial susceptibility concluded that the available evidence for most bacteria was either poor or non-existent and that the published evidence/knowledge base did not support the use of WGS-ASP to guide clinical decision making [1]. Here, we have reviewed the literature in a similar approach to assess the progress since 2017 and the current status of WGS-ASP.

A critical priority raised in 2017 was the need for an expansion in the evidence base. Since then there has been a significant increase in our understanding of the genotype–phenotype associations as a consequence of more widespread use of WGS, advancement in long-read sequencing technology, development of bioinformatic tools and resistance gene/mutation databases, and development and usage of AI/ML approaches. This has also further revealed that mechanisms such as gene copy number, derepression of intrinsic mechanisms, allelic variation, lineage variation as well as species variation play a role. Thus, our understanding of the complexity and interplay of mechanisms influencing the genotype–phenotype relationship has greatly increased. However there remains a huge disparity in the quantity and quality of evidence available for different organisms and agents, and a lack of standardization and aggregation of genotype–phenotype data.

The 2017 report divided the organisms in three groups based on the available evidence with only *S. aureus* and *M. tuberculosis* classified in the first group, where most was known. The second group included Enterobacterales, where major gaps were identified, and the third one *S. pneumoniae*, *N. gonorrhoeae*, *P. aeruginosa*, *A. baumannii* and *C. difficile* for which further studies were clearly needed. As summarized in Table 5 this has now changed and for many species-antimicrobial agent combinations the correlation between WGS-based detection of resistance genes/mutations and AST demonstrates a high-level of accuracy to distinguish WT and non-WT populations. Consequently, WGS-ASP based on ECOFF values shows high predictability for several additional organisms and agents. Still, resistance mechanisms driven by mutational overexpression of intrinsic resistance genes, such as the AmpC  $\beta$ -lactamase or efflux pumps continue to be challenging despite efforts. Gaps also frequently include new agents where there are limited combined WGS and AST data and where we are still learning the mechanisms of resistance. Moreover, the introduction of novel antimicrobial agents, and particularly novel BL/BLI combinations, is driving the emergence of novel resistance mechanisms that affect these but also older agents, thus determining the

**Table 5**  
State of the art of WGS-ASP for the targeted microorganisms.

| Microorganism          | Current WGS-ASP evidence (Low, Medium, High) | Major advances since 2017                                                                                                                                                                                                                                                                     | Remaining challenges                                                                                                                                                                                                                                                                  |
|------------------------|----------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Enterobacterales       | High                                         | Development of species-specific tools. Large scale studies elucidating the phenotype –genotype relationship of specific species-drug combinations.                                                                                                                                            | Quantitative impact and combinatorial effects of resistance mechanisms. Novel agents. Tools to identify gene-copy number effects. Species and lineage effects. Phenotypic effect of allelic variations in resistance genes. Incorporating genetic context into prediction algorithms. |
| <i>P. aeruginosa</i>   | Medium                                       | Increased understanding of mutational resistance. Tools for WGS-ASP developed.                                                                                                                                                                                                                | Quantitative impact and combinatorial effects of resistance mechanisms. Novel agents. Phenotypic effect of allelic variations in resistance genes.                                                                                                                                    |
| <i>A. baumannii</i>    | Medium                                       | Emergence of WGS coupled with ML. Used advanced model of deep learning.                                                                                                                                                                                                                       | Limited robustness (need for standardization, well-characterized isolate collections). Impact of insertion sequences. Novel agents.                                                                                                                                                   |
| <i>N. gonorrhoeae</i>  | Medium                                       | Large datasets with genomes and antibiotic resistance phenotypes enabling discovery of resistance determinants and genotype-based AMR prediction test characteristics.                                                                                                                        | Improving sensitivity and specificity of WGS-ASP. Novel agents.                                                                                                                                                                                                                       |
| <i>S. aureus</i>       | High                                         | Several bioinformatic tools have been successfully applied and improved resistance prediction.                                                                                                                                                                                                | Predictive capacities may be improved by ML and transcriptomics research.                                                                                                                                                                                                             |
| <i>S. pneumoniae</i>   | Medium                                       | Advances in MIC-prediction of $\beta$ -lactams and standard AST prediction of non- $\beta$ -lactams                                                                                                                                                                                           | Lack of reference methodology in comparisons, lack of assessment with the new breakpoints for benzylpenicillin                                                                                                                                                                        |
| <i>C. difficile</i>    | Low                                          | More WGS studies available, although still limited compared with other pathogens.                                                                                                                                                                                                             | Limited availability of WGS and AST data because of challenging growth conditions and standardization                                                                                                                                                                                 |
| <i>M. tuberculosis</i> | High                                         | WHO mutation catalogue<br>WGS-ASP replaced most routine phenotypic AST for first-line drugs in several well-resourced countries                                                                                                                                                               | Need for reference method phenotypic AST data for developing mutation catalogue especially for new drugs<br>Maintain phenotypic AST (ideally MIC testing) despite increasing adoption of WGS-ASP                                                                                      |
| Enterococci            | Medium                                       | Large scale studies elucidate that good phenotype –genotype correlations exist for standard therapeutics. Software tools for genomic prediction of resistance to linezolid exists ( <a href="https://cge.food.dtu.dk/services/LRE-finder/">https://cge.food.dtu.dk/services/LRE-finder/</a> ) | Prediction for rather multifactorial (daptomycin) and novel or unclear molecular causes of antibiotic resistances (lipoglycopeptides, tigecycline) remain to be established.                                                                                                          |
| <i>H. influenzae</i>   | Medium                                       | Correlation of different $\beta$ -lactam phenotypes, including PBP3 mutations, with WGS-ASP                                                                                                                                                                                                   | Test inferences in strains from different geographical areas                                                                                                                                                                                                                          |
| <i>B. fragilis</i>     | Low                                          | Improved prediction of metronidazole, clindamycin and, to some extent, carbapenems                                                                                                                                                                                                            | Perform correlations with non- $\beta$ -lactam antimicrobials<br>Assess correlations with other antimicrobials                                                                                                                                                                        |

AMR, antimicrobial resistance; AST, antimicrobial susceptibility testing; ML, machine learning; WGS-ASP, whole genome sequencing antimicrobial susceptibility prediction.

need for continuous refining of resistance genotype databases and phenotypic correlations. This includes for instance the importance of PBP mutations and allelic variations in  $\beta$ -lactamases involved in resistance to new BL/BLI combinations, the role of iron receptors in cefiderocol susceptibility, mechanisms of resistance to gepotidacin and zoliflodacin in *N. gonorrhoeae*, and daptomycin and tigecycline in enterococci. Thus, generation of combined WGS-AST data for newer agents is important and should not only include NWT, but also WT isolates.

WT organisms in theory should lack acquired resistance genes but this is not always the case as described above and in Yee *et al.* [14] and WGS of WT isolates is required to further delineate this relationship if WGS-ASP is to be used for WT/NWT prediction.

The main challenge of WGS-ASP is that guidance of clinical decision making based on AST requires interpretation of a determined MIC or zone diameter according to clinical breakpoints, which can deviate from the ECOFF distinguishing WT from non-WT. Thus, clinically susceptible isolates by phenotypic AST may have a resistance genotype (carry a resistance gene or mutation). There are multiple examples of this including the presence of carbapenemases in Gram-negatives and carbapenem

susceptibility or single chromosomal mutations in *gyrA* or *parC* resulting in reduced susceptibility to ciprofloxacin, but not clinical resistance. This was also highlighted in the 2017 report where clinical breakpoints should be considered a secondary comparator [1]. The advancement in AI and ML where some ML models have shown promising results in predicting MICs to a high level of accuracy should be further explored [93,238]. However, WGS and computational approaches still cannot yield an inferred MIC or zone diameter to the required extent and accuracy. Conversely, the usefulness of detecting genetic markers as direct predictors of clinical outcome regardless of MIC values should be further investigated.

Despite the improvements in recent years, many published works still fail to consider appropriate methodological approaches for the evaluation of WGS-ASP tools. WGS-ASP with the purpose of S-I-R-classification should be assessed with CA, and should employ the same rules as are generally applicable for assessment of phenotypic methods (<https://www.iso.org/obp/ui/en/#iso:std:iso:20776:-2:ed-2:v1:en>). Comparison should be done with MICs determined by reference methodology, not surrogate methods. If the intention is to predict MICs, the authors should analyse EA and

bias. The strain collection used should contain a high proportion of challenging strains including isolates with MICs on the border between susceptibility and resistance.

*M. tuberculosis* stands out as the organism where the use of genotypic assays and WGS-ASP has advanced the most and where it is now increasingly being used in routine diagnostics. A key reason supporting this is the development of the WHO mutation catalogue standardizing interpretation including assessment of the evidence grade and level of resistance for some agents [73]. However, there are still challenges ahead including further expansion of the catalogue and assessment of genotype–phenotype relationship using the reference broth microdilution method that was endorsed by EUCAST in 2019 [265].

Standardization and harmonization of analytical tools and interpretive criteria was recommended as important for further advancement in the 2017 report [1]. This is key for a unified reporting of genotypes in a similar manner as WT vs. NWT are based and interpreted according to ECOFFs and MIC and zone diameters are interpreted according to clinical breakpoints. In addition to the WHO mutation catalogue for *M. tuberculosis*, interpretive standards are now also being developed for other organisms through the ESCMID Study Group on Epidemiological Markers AMR working group (<https://github.com/AMRverse/AMRrules>). Here, organism-specific interpretation is being defined and curated for AMR genotypes that can be applied using the AMRrules tool. Moreover, to overcome the challenge of different outputs by the AMR prediction tools and the underlying databases the PHA4GE working group have developed the hAMR-ronization tool that can harmonize the output from multiple tools [88]. In addition, tools are developed facilitating the analysis of combined genotype and phenotype data (<https://amrverse.github.io/AMRgen/index.html>). These efforts are key for advancing the standardization, benchmarking and comparative analysis of tools and databases and analysis of genotype–phenotype relationships.

After this, implementation of WGS-ASP in clinical diagnostics will require compliance with IVDR/FDA and international quality standards. Some advancements have been made in this aspect with ISO-accredited workflows being developed in the public health setting [36,278, <https://gitlab.uzh.ch/appliedmicrobiologyresearch/immense>]. However, the continuous development of sequencing technology and bioinformatic tools challenges this. Furthermore, development and establishment of EQAs, PT or ring-trials is also paramount for implementation in clinical diagnostics.

Despite the advancement in sequencing technology and reduction in cost, the cost and speed compared with standard AST methods limits the implementation in clinical diagnostics. In addition, cost-benefits of using WGS-ASP and impact on clinical outcomes need further evaluation and may be context-specific.

In conclusion, there have been significant advances in many of the challenges and recommendations outlined in the previous report resulting in a step-change in our understanding of the genotype–phenotype relationship, improvements in bio-informatic tools, AMR databases, standardization and harmonization. There is now increased evidence for using WGS-ASP with ECOFF as primary comparator to distinguish WT and non-WT. However, except for *M. tuberculosis* the current evidence still does not support the paradigm shift of applying WGS-ASP to guide clinical decision making for most organism-antimicrobial agent combinations.

## Credit authorship contribution statement

O.S, C.L.C, C.G.G and A.O Conceptualization, supervision and validation. All authors writing original draft, review and editing.

## Transparency declaration

### Potential conflict of interest

C.U.K. is a consultant for the TB Alliance (New York, NY, USA), the WHO Department for HIV, TB, Hepatitis, and STIs (Geneva, Switzerland), and the WHO Regional Office for Europe (Copenhagen, Denmark). C.U.K. worked as a consultant for FIND (Grand-Saconnex, Switzerland) and the Stop TB Partnership (Geneva, Switzerland). C.U.K.'s past consulting for Becton Dickinson (Sparks, MD, USA) involved a collaboration with Janssen (Beerse, Belgium) and Thermo Fisher Scientific (Waltham, MA, USA). C.U.K. collaborated with PZA Innovation (Baltimore, MD, USA). C.U.K. is or was an unpaid advisor to Bigtec Labs (Bengaluru, India), Cepheid (Sunnyvale, CA, USA), Genoscreen (Lille, France), and Otsuka (Munich, Germany)—GenoScreen covered related travel and accommodation expenses only. C.G.G. has worked as a consultant for 1928 Bioinformatics (Gothenburg, Sweden). A.E. is a founding member and executive head of the Swiss Pathogen Surveillance Platform ([www.spsp.ch](http://www.spsp.ch)). N.S. has received research funds from Sanofi/J&J (2025). R.C. is clinical data coordinator of the European Committee of Antimicrobial Susceptibility Testing (EUCAST). All other authors have no conflicts of interest.

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## Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work, no AI tools were used.

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