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Next-generation rapid phenotypic antimicrobial susceptibility testing

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Slow progress towards implementation of conventional clinical bacteriology in low resource settings and strong interest in greater speed for antimicrobial susceptibility testing (AST) more generally has focused attention on next-generation rapid AST technologies. In this Review, we systematically synthesize publications and submissions to regulatory agencies describing technologies that provide phenotypic AST faster than conventional methods. We characterize over ninety technologies in terms of underlying technical innovations, technology readiness level, extent of clinical validation, and time-to-results. This work provides a guide for technology developers and clinical microbiologists to understand the rapid phenotypic AST technology landscape, current development pipeline, and AST-specific validation milestones.

Inadequate access to clinical bacteriology testing in low resource settings (LRS) impedes management of individual patients, detection of antimicrobial resistance (AMR), and implementation of effective antimicrobial stewardship (AMS) interventions[1–3]. This fuels the overuse of empiric antimicrobials driving AMR, now among the greatest threats facing humanity[4]. AMR disproportionately affects low-income countries, particularly those in sub-Saharan Africa and South Asia[4]. Unfortunately, only 1.3% of 50,000 medical laboratories in 14 sub-Saharan countries offered any clinical bacteriology testing as of 2019, owing to multiple factors that thwart scale-up of conventional bacteriology[5]. These include a requirement for specialized infrastructure, a lack of automation, and inadequate local access to a complex supply-chain which is compounded by foreign exchange distortions in many countries[3,6]. Diagnostic sectors focused on single diseases like HIV, tuberculosis, malaria, or COVID-19 require a comparatively short list of platforms or supplies[7,8]. In contrast, clinical bacteriology laboratories (CBL) require a complex supply chain, which can involve hundreds of stable-sourced and quality-assured components. Though frequently inexpensive, they are often unavailable in LRS or cannot be purchased without foreign currency, which itself is often inaccessible. Human resource challenges and a relative lack of granular guidance further complicate CBL implementation.

The cornerstone of clinical bacteriology laboratories is diagnosis of bloodstream infections[9]. This conventionally involves three sequential processes: detection of bacterial growth, taxonomic identification of isolated bacterial colonies, and antimicrobial susceptibility testing (AST). Detection of bacterial growth in blood culture bottles can take up to 5 days, but often occurs within the first 24 h of incubation[10,11]. Next, bacterial identification typically takes another 24 h. Finally, AST on pure bacterial colonies typically also requires 4–24 h. Nucleic acid amplification tests (NAAT) have been proposed to accelerate this process. Alas, most genotypic methods currently rely on detecting a limited number of targets in a manner that is neither hypothesis-free nor allows for the detection of the diverse AMR mechanisms across clinical settings. For example, a carbapenemase gene is identifiable in fewer than 50% of bacteria found to be phenotypically carbapenem resistant[12]. Thus, despite considerable contributions of NAAT-based diagnostics in the fields of tuberculosis, HIV,

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and malaria, few available technologies have emerged so far as a suitable replacement for conventional bacteriology to yield rapid and accurate AST. Lack of progress towards implementation of conventional clinical bacteriology in low resource settings, as well as interest in greater speed and accuracy for AST more generally, has shifted attention toward next-generation, rapid AST technologies.

In this Review, our aim was to understand the current pipeline of AST technologies and what role they may play in bridging the diagnostic gap currently facing LRS. We performed a scoping review of scientific publications and submissions to regulatory agencies describing technologies capable of providing phenotypic AST in a shorter timeframe than conventional methods. We then characterized the pipeline of identified AST technologies in terms of their underlying technical innovations, technology readiness level (TRL), extent of clinical validation, and standardized expected time-to-results from specimen collection.

Methods

Search strategy

We searched PubMed using the MeSH search terms "Microbial Sensitivity Tests" [MeSH]; "Rapid Diagnostic Tests" [MeSH], "Phenotype" [MeSH] and non-MeSH keywords "antimicrobial sensitivity", "antimicrobial susceptibility", "rapid", "phenotyp*". The search was last updated in July 2024, and articles from 1946 to present were included. No language limit was applied. We first screened titles and abstracts for relevant information and then used a snowball search strategy to screen reference lists to retrieve other relevant articles.

The FDA 510(k) Premarket Notification and FDA Premarket Approval (PMA) databases were searched with the Classification Product Codes "LON", "JWY" and "LRG" to find tests falling under the categorization of "system, test, automated, antimicrobial susceptibility, short incubation", "manual antimicrobial susceptibility test systems", "instrument for auto reader & interpretation of overnight susceptibility systems" respectively. When a device had multiple submissions, the most recent submission that provided details on the organisms included in the study design was used. We also performed Google searches with the terms "rapid phenotypic antimicrobial susceptibility test". Finally, several content experts in microbiology and bio/biomedical engineering were consulted to probe for lacunes in our search strategy. When we identified a technology missed by our search, we adapted it to ensure it was captured by the above strategy. We considered as "commercialized" any AST technology with any of the following: FDA authorization, approval, or a pre-market notification; European Economic Area CE marking; or authorization by another WHO-Listed Authority (WLA) if specified by authors. All others were considered "non-commercialized".

Rapid AST platforms were included if they relied on phenotypic antimicrobial susceptibility profiling of bacteria, regardless of the recognition element used. Rapid technologies were defined as those offering a faster time-to-final-result than those possible with conventional clinical microbiology methods. The latter typically require a minimum of 72 h from specimen collection to final susceptibility results and at least 4 h after the isolation of pure bacterial colonies[13]. Phenotypic tests were defined as those that measure microbial growth or viability in the presence of antimicrobials to determine susceptibility. Hypothesis-free nucleic acid-based tests were defined as those using genomic recognition elements to detect or quantify bacteria in the presence of different antimicrobial conditions without pre-defined targets. Although they represent a subgroup of "phenotypic tests", we considered methods using nucleic acid-based recognition elements in a distinct category of technologies to facilitate comparison between them. We only included technologies with publications that specifically addressed their application to phenotypic AST. Finally, we only considered technologies used for non-mycobacterial vegetative bacteria routinely isolated in clinical laboratories.

Data extraction

All titles, abstracts, and full texts in PubMed and FDA databases were screened by one reviewer (GR). In addition, an independent microbiologist reviewer (KH) audited all included publications or technologies and extracted data. Data extracted included relevant information on bacterial testing (including number, source, and species of bacterial isolates, as well as species-antimicrobial combinations tested), specimen characteristics (specimen matrix, number of patients, retrospective/prospective collection), performance metrics as outlined by the Clinical and Laboratory Standards Institute (CLSI) (minimum inhibitory concentration [MIC], essential agreement, categorical agreement, minor and major errors), time-to-result, and a description of the technology.

Identified technologies and comparative frameworks

Number of identified platforms

We identified 81 publications describing non-commercialized AST platforms (67 phenotypic and 14 hypothesis-free nucleic acid-based). We also identified 18 commercialized platforms, with 12/18 having FDA 510(k) clearance and CE marking and 6/18 having only CE marking. Eight commercialized platforms were described in 34 publications either pre- or post-commercialization. Nine technologies were identified external to our literature search in PubMed and the FDA databases via consultation with experts and internet searches (PRISMA diagram, Fig. 1).

Technology Readiness Level for antimicrobial susceptibility testing technologies

To understand how close non-commercialized technologies are to commercialization, we created an AST Technology Readiness Level framework (Table 1) adapted from the general Technology Readiness Level (TRL) frameworks from the Government of Canada[14] and US Department of Health and Human Services[15]. We then described the key elements of each commercial, non-commercial phenotypic, and non-commercial hypothesis-free nucleic-acid based technology and classified all identified non-commercial technologies according to the TRL framework (Tables 2, 3, and 4).

Extent of clinical validation of technologies

While the TRL is designed to broadly stage the development of new technologies, it does not provide a direct understanding of the extent of clinical validation or use-case assessment of a given system[16]. The FDA provides guidelines on the study design necessary for regulatory approval, but early-phase studies are difficult to compare across publications. In contrast to the case for pharmaceutical therapeutics[17], no standardized framework currently classifies the diversity of clinical validation studies of AST platforms. Consequently, we developed a framework to type the full spectrum of AST diagnostic studies, building on previous work for diagnostic studies in general[18,19]. This framework is meant to help to understand the AST diagnostics landscape and may serve to guide subsequent work. Figure 2 shows the Phase of Clinical Validation framework and how studies identified in by our search strategy map onto it.

Evaluation of Turn-Around-Time (TAT) from the time of specimen collection

We defined the time interval from clinical specimen collection (e.g. collecting blood or urine from a patient) to final AST results as the most meaningful metric of a test's Turn-Around-Time (TAT). This is a key parameter to predict the value of novel rapid AST platforms because delayed administration of effective antimicrobials has been repeatedly associated with increased mortality among patients with severe manifestations of sepsis[20–22]. Reports describing technologies we identified frequently cited time-to-result from a different starting

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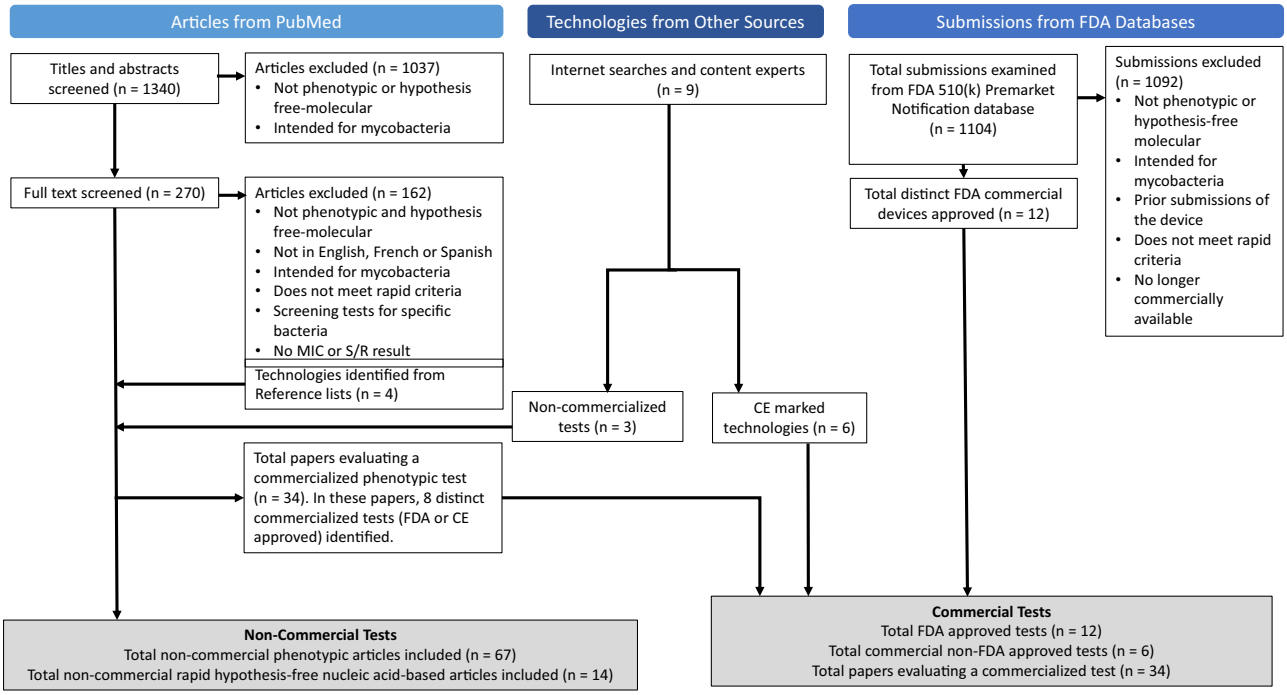

Fig. 1 | PRISMA flow diagram of search strategy. A systematic search strategy encompassed PubMed and the FDA 510(k) Premarket Notification and FDA Premarket Approval (PMA) databases. Iterative internet searches and consultation with content experts in microbiology and bio/biomedical engineering were used to probe for lacunes in our search strategy. Technologies were included if they relied on phenotypic antimicrobial susceptibility profiling of bacteria, regardless of the recognition element used. Rapid technologies were defined as those offering a faster time-to-final-result than those possible with conventional clinical microbiology methods. Phenotypic tests were defined as those that measure microbial growth or viability in the presence of antimicrobials to determine susceptibility. Hypothesis-free nucleic acid-based tests were defined as those using genomic recognition elements to detect or quantify bacteria in the presence of different antimicrobial conditions without pre-defined targets. We considered methods using nucleic acid-based recognition elements in a distinct category of technologies to facilitate comparison between them. We only included technologies with publications that specifically addressed their application to phenotypic AST. Finally, we only considered technologies used for non-mycobacterial vegetative bacteria routinely isolated in clinical laboratories. We considered as "commercialized" any AST technology with any of the following: FDA authorization, approval, or a pre-market notification; European Economic Area CE marking; or authorization by another WHO-Listed Authority (WLA) if specified by authors. All others were considered "non-commercialized".

point than specimen collection, opacifying direct comparison of claimed TAT between different technologies.

To standardize TAT reporting and provide a level playing field for inter-technology comparisons, we devised a methodology using evidence-based assumptions regarding the average time from specimen collection to a positive blood culture and from specimen collection to pure colony isolation. Figure 3 depicts a standard clinical microbiology workflow and how the TAT of identified technologies align with it. The assumed delays for each step are in line with the daily workflow used in most clinical microbiology laboratories. However, shorter overall TAT are possible with conventional techniques if a continuous workflow eliminates delays caused by "dead time" (i.e. if each step was initiated at the earliest possible moment for each specimen). In practice, this is possible only with fully robotic automation which is deployed in a small number of laboratories at very high initial cost. Most technologies we identified relied on a combination of innovations to reduce overall TAT rather than a single element (Fig. 4).

Understanding the current rapid AST technology landscape and pipeline

We extend the work of prior narrative reviews by using a rigorous scoping review methodology to perform a comprehensive and reproducible search, encompassing both peer-reviewed scientific literature and submissions to regulatory agencies, to provide an up-to-date understanding of the landscape of available rapid AST technologies and the development pipeline[23–25]. We also expand on the 26 technologies identified in a 2019 landscape analysis on simplified blood culture systems from the Foundation for Innovative New Diagnostics (FIND)[26]. We identified 120 publications describing over 90 rapid AST technologies promising TAT faster than that currently possible with conventional clinical microbiology methods (81 reporting non-commercialized technologies and 34 describing 9 of the 18 commercially available products). Among non-commercialized technologies, a wide array of phenotypic and hypothesis-free nucleic acid-based AST methods were identified. We proposed AST-adapted frameworks to evaluate and compare *Technology Readiness Level* and *Phase of Clinical Validation* for non-commercialized technologies to respectively capture the stage of development of the technology and the extent of published validation with bacterial isolates and clinical specimens. All 18 commercially available technologies were also assessed using the *Phase of Clinical Validation* framework to characterize the evidence base available for their clinical use.

Commercialized rapid phenotypic AST technologies

Most of the 18 commercialized rapid phenotypic AST technologies we identified combine multiple innovations to shorten TAT (Table 2). Nine commercialized technologies or alternative methods produced by standards organizations circumvent the need to isolate bacteria in pure colonies by providing phenotypic results directly from positive blood culture bottles after a necessary incubation period[27–36] (Fig. 3, Table 2). This reduces TAT by at least 24 h compared to conventional practices. Among these, only 5/9 have undergone Phase 4 clinical validation studies as identified in our search, reflecting that such technologies are yet to be widely evaluated in routine clinical use. Notably, one commercialized technology with regulatory approvals and clinical validation data offers direct-from-specimen phenotypic

Table 1 | Technology Readiness Levels adapted for rapid antimicrobial susceptibility tests (AST)

Level	Description	Example
1	**Basic Principles Observed and Reported** Active monitoring of scientific knowledge base to identify phenomenon and/or pathological markers to assess bacterial viability.	Bacteria is observed to produce heat when viable.
2	**Technology Concept and/or Application Formulated** Hypothesis generated for how a phenomenon could be used in an antimicrobial susceptibility test with none-to-minimal development of an experimental proof of concept.	A microcalorimeter is proposed to measure the amount of bacterial metabolism as a measurement of growth.
3	**Analytical and Experimental Critical Function and/or Proof of Concept** Active research and design have begun, and a basic proof of concept model is developed without integration of components into a complete system.	A microcalorimeter is built to measure heat production of bacteria.
4	**Component Validation in a Laboratory Environment** Multiple component pieces are tested together, and a plan is developed for critical design requirements.	A pre-existing antibiotic gradient generator is combined with the novel microcalorimeter and tested with bacterial isolates. Plans are made for how urine sample processing will be integrated into the device.
5	**Component Validation in a Simulated Environment** Spiked sample matrices and/or a small number of clinical samples are tested on components.	Spiked urine samples and a small number of clinical urine samples are tested on the pre-existing antibiotic gradient generator and novel microcalorimeter.
6	**Complete System Demonstration in a Simulated Environment** A fully functional prototype is built and tested with spiked samples and/or a small number of patient samples.	Sample processing techniques, antibiotic gradient generation and a microcalorimeter are all integrated into one device and tested with spiked urine and/or a small number of patient samples.
7	**Complete System Demonstration in an Appropriate Operational Environment** A fully functional prototype is demonstrated in an appropriate environment that includes many patient samples, bacterial strains, and antibiotics.	A fully functional prototype is tested with numerous patient samples and bacterial strains.
8	**Actual Technology Completed and Qualified through Tests and Demonstrations** Technology is ready for regulatory approval submission. Studies have been performed with sufficient susceptible, resistant, and challenge organisms from clinical samples and quality control organisms. Studies also include comparison to a reference method, demonstrated reproducibility, and acceptable performance characteristics.	Studies have been conducted that meet FDA class II requirements for regulatory approval and a submission has been made to the FDA.
9	**Actual Technology Proven through Successful Deployment in an Operational Setting** Regulatory approval has been acquired and device is ready for marketing.	FDA approval has been granted.

This framework provides a metric to evaluate the stages of technological development and how close a rapid AST technology is to commercialization. FDA Class II requirements for rapid AST technologies are taken from the reference cited[80].

AST without pre-incubation (PA-100 AST System, SYSMEX Europe SE), though only from urine specimens at this time. This is significant because while final AST results for urine cultures are conventionally available within 48 h of collection, the ~ 44 hr reduction in TAT offered by this method opens the possibility of new use cases at the point of need. Nearly all commercialized technologies we identified represent refinements of microbiology processes that must already be implemented in the first place. For low resource settings without conventional bacteriology services, this means that most such technologies may be very useful but do not inherently provide a means of leapfrogging current obstacles to implementing bacteriology in low- and middle-income countries (LMIC). We have highlighted potential advantages and limitations to implementation of each commercialized technology, recognizing that LMIC versus high-income countries may face different barriers.

The pipeline of non-commercialized rapid phenotypic AST technologies

Methods most frequently employed to decrease time-to-AST results by non-commercialized platforms included novel detection elements, single-cell imaging, miniaturization of growth chambers, and microfluidic assay designs, usually in combination with a minimized incubation time (Tables 3 and 4). However, the majority of these rapid phenotypic AST technologies offered only incremental reductions in TAT compared to the modern clinical microbiology laboratory workflow (Fig. 3). Markedly faster non-commercialized technologies were described in six publications reporting phenotypic results directly from positive blood culture bottles—i.e. blood that has been incubated to culture-amplify infecting bacteria until bacterial growth is detected by an automated system—either clinically obtained positive blood culture specimens ($n = 1$) or sterile blood spiked with bacterial isolates and then incubated for culture-amplification ($n = 5$)[37–41] (Fig. 3, Tables 3 and 4). The technology reporting AST directly from clinically obtained positive blood culture bottles utilizes a microfluidic assay design with integrated antimicrobial content similar to existing commercial technologies but adds an additional centrifugation component to concentrate the specimen for optimal growth and light microscopy-based imaging[37]. For reference, two recently commercialized technologies capable of direct-from-positive blood culture bottles results (QuickMIC from Gradientech and dRAST from QuantaMatrix) proceeded from pre-commercialization publications to European market approval in 6–7 years[42–45]. Another recently published technology provides results for both species identification and AST directly from patient blood specimens without use of culture-amplification in blood culture bottles; this technology uses a novel bacterial isolation step prior to proceeding, demonstrating a significant advancement in managing a more complex specimen matrix[46].

We also identified 7 non-commercialized technologies reporting direct-from-specimen phenotypic AST via MIC measurement. One technology describes AST performed directly from a blood specimen, without need for incubation in a blood culture bottle, and incorporates simultaneous species identification[46]. The other six of these all used a urine matrix, with 1/6 also provided data using a blood matrix[47–53] (Fig. 3, Tables 3 and 4). All were reported in 2020 or later, with advanced TRL and well-described studies using clinical specimens in 3/7[50,51] and 1/7 having a moderate-sized prospective clinical study (Phase 2b)[51]. For the moment, a preponderance of data for direct-from-specimen AST platforms reports performance data for only a few

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Table 2 | Description of phenotypic, commercially available methods of antimicrobial susceptibility testing

Technology principles	Detailed technology description	Commercial name	Company or organization	Advantages[a]	Limitations[a]
Macroscopic growth measured visually	Direct visualization of bacterial growth on solid media following exposure to antimicrobial gradient strips	**ETEST®**[81]	bioMérieux SA	No instruments required Low barrier to implementation	Pure culture required Skilled interpretation required
	Direct visualization of bacterial growth on solid media following exposure to antimicrobial gradient strips	**MTS™ (MIC Test Strip)**[82]	Liofilchem S.r.l.	No instruments required Low barrier to implementation	Pure culture required Skilled interpretation required
	Direct visualization of bacterial growth on solid media after exposure to antimicrobial discs; plates inoculated directly from positive blood cultures	**Rapid AST directly from positive blood cultures**[27]	EUCAST	No instruments required Direct from positive blood culture	Not suited to laboratories with fixed daily workflow Results are preliminary only – final AST results using conventional methods required Currently validated for 8 species only
Bacterial concentration detected via light absorbance	Directly read or automated broth microdilution available in pre-formatted cassettes	**ComASP®**[83]	Liofilchem S.r.l.	No instruments required	Pure culture required
	Automated broth microdilution	**VITEK® 2**[84]	bioMérieux SA	Automated; part of extensive instrument ecosystem	Pure culture required Manual dilution steps required
	Automated broth microdilution using metabolic indicator dye and light absorbance measurements	**BD Phoenix™**[85]	Becton Dickinson and Company	Automated; part of extensive instrument ecosystem	Pure culture required Manual dilution steps required
	Visualization of bacterial concentration during exposure to antimicrobials using light absorbance measurements; presence of indicator dye not specified	ASTar®[28]	Q-linea AB	Does not require manual dilution steps	Pure culture required
Bacterial concentration detected via fluorescence production	Automated broth microdilution using fluorescence detection and universal fluorescent dye	**Sensititre™**[86]	Thermo Fisher Scientific, Inc.	No instruments required Customizable choice of antimicrobials	Pure culture required Manual dilution steps required
	Visualization of bacterial concentration during exposure to antimicrobials using fluorescence detection and universal fluorescent dye	**DxM MicroScan Walk-Away ID/AST System**[87]	Beckman Coulter, Inc.	Automated; part of extensive instrument ecosystem	Pure culture required Manual dilution steps required
	Visualization of proportion of viable bacteria during exposure to antimicrobials using flow cytometry and universal fluorescent dyes with single-cell resolution	FASTinov AST[29]	FASTinov S.A.	Direct from positive blood culture Rapid TAT of 2 h from positive blood cultures	Flow cytometry instrument required Requires separate workflow for species ID
Bacterial concentration detected via advanced imaging or laser-based methods	Visualization of bacterial concentration during exposure to antimicrobials using laser light scattering	Alfred 60/AST[30]	Alifax S.r.l.	Integrated liquid culture and AST system that begins AST as soon as culture ready	Requires separate workflow for species ID
	Visualization of bacterial replication during exposure to antimicrobials using microcolony-resolution light microscopy	dRAST™[31]	QuantaMatrix Inc.	Direct from positive blood culture Rapid TAT of 4 h from positive blood cultures Runs 12 samples simultaneously	Requires separate workflow for species ID
	Visualization of individual bacterial replication during exposure to antimicrobials using phase contrast microscopy within nanofluidic channels	PA-100 AST System[88]	SYSMEX Europe SE	Direct from urine specimen Rapid TAT of 45 min from sample insertion No sample preparation; designed for point-of-care use	Does not provide species ID Does not work with samples containing more than one organism Urine specimens only One sample per run
	Visualization of bacterial replication during exposure to antimicrobial gradients within a microfluidic device using single-cell resolution dark field microscopy	QuickMIC®[32]	Gradientech AB	Direct from positive blood culture Rapid TAT of 4 h from positive blood cultures Modular	Only has gram-negative AST panel available Requires separate workflow for species ID

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Table 2 (continued) | Description of phenotypic, commercially available methods of antimicrobial susceptibility testing

Technology principles	Detailed technology description	Commercial name	Company or organization	Advantages[a]	Limitations[a]
Bacterial concentration detected via physical interactions	Detection of individual bacterial mass during exposure to antimicrobials using a cantilever within microfluidic channel	LifeScale[33]	Affinity Biosensors	Direct from positive blood culture Rapid TAT of 4.5 h from positive blood cultures Runs 4 patient samples simultaneously	Only has gram-negative AST panel available Requires separate workflow for species ID
Bacterial growth measured indirectly via metabolic activity	Detection of bacterial metabolic changes during exposure to antimicrobials using microcantilever movements after bacterial attachment to cantilever	Resistell™[34]	Resistell AG	Direct from positive blood culture Rapid TAT of 2 h from positive blood cultures Runs 12 samples simultaneously	Requires separate workflow for species ID
	Detection of gaseous bacterial metabolic products during exposure to antimicrobials using colour-changing sensors monitored via camera	VITEK® REVEAL™[35]	bioMérieux SA	Direct from positive blood culture Rapid TAT of 6 h from positive blood cultures Modular	Requires separate workflow for species ID
Bacterial concentration and/or activity measured via multiple methods	Visualization of bacterial replication during exposure to antimicrobials using both fluorescence and dark field microscopy with universal rRNA probes; image processing via morphokinetic cellular analysis (incorporates multiple imaging features of growth at single-cell resolution); performs automated removal of sample matrix via gel electrophoresis prior to assay	Accelerate Pheno®[36]	Accelerate Diagnostics, Inc.	Integrated species ID Direct from positive blood culture Rapid TAT of 7 h from positive blood cultures Modular	
	Visualization of bacterial surface area during exposure to antimicrobials using both fluorescence microscopy and charge-based fluorescent dye with specific binding to bacterial surface as well as visualization of metabolic activity using a metabolic indicator dye and light absorbance measurements	Selux Next Generation Phenotyping System[89]	Selux Diagnostics, Inc.	Automated Direct from positive blood culture Rapid TAT of 7 h from positive blood cultures	Requires separate workflow for species ID

Commercial names in bold denote methods in wide use[90,91]. [a]Key advantages and limitations of commercial technologies are outlined, with a focus on implementation considerations, user experience, and clinical use cases. While important, we did not include economic considerations as these are beyond the scope of our review and are highly context-dependent. Several technologies require complex instruments and workflows; they also frequently require an ecosystem of supporting technologies for AST-related functions such as species identification without which MIC data is not interpretable. Technologies with a low barrier to implementation and/or integrated species identification are indicated in the Advantages column.

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Table 3 | Direct imaging methods for phenotypic antimicrobial susceptibility testing

Technology principles	Notable features of technology principle[a]	Detailed technology description	Quantitative MIC result	First author	Publication year	Rapid AST TRL	Phase of clinical validation
Bacterial concentration detected via light absorbance	Advantages: Low barrier to implementation. Disadvantages: Test results interpreted manually may be subject to inter-reader variability; often skilled interpretation is required.	Visualization of bacterial biomass after exposure to antimicrobials, and centrifugation in a capillary tube to allow visual or smartphone-based measurement	Yes	*Chen*[92]	2024	4	1a, 1b
		Visualization of bacterial replication during exposure to antimicrobials using a metabolic indicator dye and light absorbance measurements via iPhone	No	*Kadlec*[93]	2014	5	1c
		Standard broth microdilution protocol using shortened incubation period	Yes	Carneiro[94]	2023	4	1b
		Visualization of bacterial replication during exposure to linezolid using pH-sensitive indicator dye and macroscopic assessment of colour	No	Sordo[95]	2023	4	1b
		Visualization of bacterial replication during exposure to antimicrobials within nanowells using light absorbance measurements	Yes	Veses-Garcia[96]	2018	4	1b
Bacterial concentration detected via fluorescence production	Advantages: Fluorescent probes can enable single cell resolution and high sensitivity. Disadvantages: Requires specialized instruments for producing the excitation wavelength and measuring the emission. Universal fluorescent dyes/probes need organism identification in parallel and may be confounded by other bacteria or living organisms in the sample.	Visualization of bacterial replication during exposure to antimicrobials using fluorescence microscopy and universal fluorescent dye with single-cell resolution	No	*Toosky*[49]	2020	7	1a, 2b
		Visualization of bacterial metabolic activity during exposure to antimicrobials via microfluidics using a metabolic indicator dye and fluorescence microscopy	Yes	Nguyen[97]	2023	6	1b, 2a
		Visualization of bacterial concentration following exposure to antimicrobials using flow cytometry and universal fluorescent dye	Yes	*Velican*[50]	2020	6	1a, 2a
		Visualization of bacterial replication during exposure to antimicrobials using fluorescence microscopy and universal fluorescent dye within nanodroplet array of a microfluidic device	No	*Avesar*[47]	2017	6	2a
		Visualization of bacterial growth following exposure to antimicrobials within a microwell plate using a luciferin/luciferase-based bioluminescence signal	Yes	Sever[98]	2024	4	1b
		Visualization of bacterial concentration following exposure to antimicrobials using flow cytometry and universal fluorescent dye	Yes	Kallai[99]	2021	4	1a
		Visualization of proportion of viable bacteria during exposure to antimicrobials using flow cytometry and universal fluorescent dyes with single-cell resolution	Yes	Sawada[100]	2021	4	1a
		Visualization of individual bacterial replication during exposure to antimicrobials using fluorescence microscopy and universal fluorescent dye within microfluidic nanogap	No	Busche[101]	2019	4	1a
		Visualization of bacterial surface area during exposure to antimicrobials using fluorescence microscopy and charge-based fluorescent dye with specific binding to bacterial surface	Yes	Flentie[102]	2019	4	1a
		Visualization of bacterial growth (both replication and cell enlargement) during exposure to antimicrobials in microdroplets using fluorescence microscopy and universal fluorescent dyes with single-cell resolution	Yes	Kang[103]	2019	4	1a

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Table 3 (continued) | Direct imaging methods for phenotypic antimicrobial susceptibility testing

Technology principles	Notable features of technology principle[a]	Detailed technology description	Quantitative MIC result	First author	Publication year	Rapid AST TRL	Phase of clinical validation
		Visualization of bacterial concentration during exposure to an antimicrobial gradient within polypropylene microfluidic device using fluorescence microscopy	Yes	Sun[104]	2019	4	1a
		Visualization of individual bacterial replication during exposure to antimicrobials using fluorescence detection and universal fluorescent dye within picodroplet array of a microfluidic device	No	Kaushik[105]	2017	4	1a
		Visualization of bacterial shear stress during exposure to antimicrobials using fluorescence and phase contrast microscopy and universal fluorescent dye with single-cell resolution within a microfluidic device	No	Kalashnikov[106]	2012	4	1a
		Visualization of bacterial concentration during exposure to antimicrobials in a microplate using a commercially available plate reader to detect fluorescence signal (uses species-specific, antibody-mediated dye)	Yes	Koskinen[107]	2008	4	1a
Bacterial concentration detected via advanced imaging	Advantages: High sensitivity. Disadvantages: Require specialized reading instruments.	Visualization of agarose-immobilized bacterial replication during exposure to antimicrobials within microwells using light microscopy; integrated species identification using DNA probe-based method; uniquely direct-from-blood (without culture step) by labelling bacteria with magnetic beads and separating them from blood matrix as first step	Yes	**Kim**[46 b]	2024	6	1c, 1d, 2a
		Visualization of bacterial replication during exposure to antimicrobials within a microfluidic device using light microscopy; initial centrifugation step used to concentrate bacteria within device	Yes	**Zhu**[37]	2023	5	1b, 2a
		Visualization of bacterial surface area following exposure to antimicrobials using scanning electron microscopy	No	Bellali[108]	2022	5	1c
		Visualization of bacterial concentration during exposure to antimicrobial gradient within a microfluidic device using dark-field microscopy	Yes	Wistrand-Yuen[109]	2020	5	1c
		Visualization of bacterial replication during exposure to antimicrobials using large volume light scattering microscopy	No	*Mo*[110]	2019	5	1a, 1b, 1c
		Visualization of agarose-immobilized bacterial replication during exposure to antimicrobials within microwells using single-cell light microscopy	Yes	Kals[111]	2024	4	1a
		Visualization of bacterial concentration during exposure to antimicrobials within microdroplets secured by oil layer using single-cell bright-field microscopy	Yes	Li[112]	2023	4	1a
		Visualization of real-time bacterial replication during exposure to antimicrobials within microfluidic traps using phase-contrast microscopy with single-cell resolution; simultaneous identification using species-specific, fluorescent ssDNA probes for 16-23S RNA	No	Kandavalli[113]	2022	4	1a

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Table 3 (continued) | Direct imaging methods for phenotypic antimicrobial susceptibility testing

Technology principles	Notable features of technology principle[a]	Detailed technology description	Quantitative MIC result	First author	Publication year	Rapid AST TRL	Phase of clinical validation
		Visualization of bacterial replication during exposure to antimicrobials using bright-field microscopy within nanodroplets in a microfluidic device	Yes	Li[114]	2022	4	1a, 1b
		Visualization of bacterial concentration during exposure to antimicrobials within a droplet-based microfluidic device using fluorescence and light microscopy	Yes	Sklavounos[115]	2021	4	1a
		Visualization of bacterial growth during exposure to antimicrobials on a novel slide-gel sandwich using phase contrast microscopy with single-cell resolution	Yes	Song[116,117]	2019	4	1a
		Visualization of bacterial concentration following exposure to antimicrobials using laser transmittance through bacteria-containing optical microfibers	Yes	Cansizoglu[118]	2019	4	1a, 1b
		Visualization of bacterial concentration during exposure to antimicrobials within an existing commercial system using laser scattering	No	Idelevich[119]	2017	4	1a, 1b
		Visualization of individual bacterial growth during exposure to antimicrobials using phase-contrast microscopy within individual microfluidic cell traps	No	Baltekin[120]	2017	4	1a, 1b
		Visualization of bacterial replication within picodroplets during exposure to antimicrobials using light scatter microscopy and subsequent droplet sorting	No	Liu[121]	2016	4	1a
		Visualization of individual bacterial motion change during exposure to antimicrobials using light microscopy and antibody-based microbial tethering	No	Syal[122]	2016	4	1a
		Visualization of bacterial replication during exposure to antimicrobials using dark-field microscopy with single-cell resolution	No	Price[123]	2014	4	1a, 1b
		Visualization of bacterial replication during exposure to antimicrobials using dark-field microscopy with single-cell resolution within a microfluidic device	No	Burnham[124]	2014	4	1a

Description of methods available in published literature but not commercially available ordered according to their Rapid AST Technology Readiness Level (TRL) (definitions in Table 1), followed by date of publication. Italicized first author indicates direct-from-urine specimen capability, bolded first author indicates direct-from-positive blood culture bottle capability, and bolded plus italicized first author indicates both direct-from-urine specimen and direct-from-positive blood culture bottle capabilities. Phase of Clinical Validation also reported (definitions in Fig. 2).

[a]The net advantages and limitations of mature diagnostic platforms almost always reflect a balance of multiple elements beyond a single underlying technology that determine overall usability.

[b]This technology uniquely demonstrates direct-from-blood specimen capability without a culture amplification step.

Table 4 | Spectroscopy-based, laser-based, and non-optical methods for phenotypic antimicrobial susceptibility testing

Technology principles	Notable features of technology principle[a]	Detailed technology description	Quantitative MIC result	First author	Publication year	Rapid AST TRL	Phase of clinical validation
Bacterial concentration detected via light spectroscopy-based methods	Advantages: High sensitivity. Disadvantages: Requires isolated colonies to not be confounded by other species in the sample.	Detection of bacterial metabolic activity during exposure to antimicrobials using change in reflected light spectrum of microfluidic cassette nanosurface in combination with resazurin reduction	Yes	*Jalali and AbdElFatah*	2024 (submitted manuscript)	6	1a, 1b, 1c, 1 d, 2b
		Visualization of bacterial concentration change during exposure to antimicrobials using changes in reflected optical signal from nano-cantilever immersed in culture medium	Yes	Bennett[125]	2020	4	1a, 1b
		Visualization of bacterial concentration during exposure to antimicrobials within a microfluidic device with diffraction grating using light spectroscopy	Yes	Heuer[126]	2022	4	1a
		Detection of bacterial concentration during exposure to antimicrobials using light diffraction signal through nanogaps within a microfluidic device	No	Busche[127]	2020	4	1a
		Detection of reflected light spectra of bacteria exposed to antimicrobials within a microfluidic device containing vertically oriented gratings	Yes	Leonard[128]	2017	4	1a
Bacterial concentration detected via laser-based methods	Advantages: High sensitivity. Disadvantages: Requires isolated colonies to not be confounded by other species in the sample.	Detection of dynamic laser scattering through liquid bacterial culture (as a measurement of replication) during exposure to antimicrobials	Yes	Zhou[129]	2020	4	1a, 1b
		Detection of laser scatter within flow cytometer (as measurement of concentration) from bacteria following exposure to antimicrobials	No	**Huang**[40]	2018	5	1c
Bacterial concentration detected via electrochemical interactions	Advantages: High sensitivity. Disadvantages: Requires isolated colonies to not be confounded by other species in the sample.	Detection of both electrochemical impedance spectrum and reduction of resazurin in bacterial culture during exposure to antimicrobials within a microfluidic device	Yes	Riester[130]	2024	5	1c
		Detection of both electrochemical impedance spectrum and microbial extracellular electron transfer in bacterial biofilm model during exposure to antimicrobials	Yes	Rafiee[131]	2024	4	1a
		Detection of electrochemical impedance spectrum (as a measurement of concentration) in bacterial culture during exposure to antimicrobials	No	Hannah[132]	2020	4	1a
		Detection of bacterial concentration during exposure to antimicrobials using antibody-mediated bacterial binding to electrodes and subsequent electrochemical current generation	Yes	**Shi**[38]	2018	4	1c
Bacterial growth measured indirectly via metabolic activity	Advantages: Reflects an in vivo process; secreted metabolites can be more abundant than individual proteins and therefore allow higher sensitivity. Disadvantages: Must be adapted to different metabolic rates of different bacterial species, metabolic activity of bacteria must not be confounded by metabolic activity of other living cells in the sample.	Visualization of catalase-positive bacterial metabolic activity during exposure to antimicrobials using catalase activity plus indicator dye and light absorbance measurements	No	*Huang*[48]	2022	6	2a
		Detection of bacterial metabolic activity during exposure to antimicrobials using deuterium labelling and Raman spectroscopy with single-cell resolution	Yes	Yi[133]	2021	6	1a, 2a

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Table 4 (continued) | Spectroscopy-based, laser-based, and non-optical methods for phenotypic antimicrobial susceptibility testing

Technology principles	Notable features of technology principle[a]	Detailed technology description	Quantitative MIC result	First author	Publication year	Rapid AST TRL	Phase of clinical validation
		Detection of bacterial metabolic activity during exposure to antimicrobials using deuterium labelling and Raman spectroscopy with single-cell resolution	Yes	Bauer[134]	2020	6	1a, 1b
		Detection of bacterial metabolic activity during exposure to antimicrobials using deuterium labelling and stimulated Raman spectroscopy with single-cell resolution	Yes	***Zhang***[53]	2022	5	1c
		Detection of current through sensor as redox-active sensor coating is oxidized by bacterial metabolites after a drop of bacterial culture briefly exposed to antimicrobials is applied	No	**Bolotsky**[41]	2021	5	1c
		Detection of current through sensor as redox-active sensor coating is oxidized by bacterial metabolites after a bacterial culture exposed to antimicrobials is applied	No	*Crane*[135]	2021	5	1c
		Detection of bacterial metabolic activity following exposure to antimicrobials using MALDI-TOF mass spectroscopy	No	**Nix**[39]	2020	5	1 d
		Detection of bacterial metabolic activity during exposure to antimicrobials using deuterium labelling and stimulated Raman spectroscopy with single-cell resolution	Yes	***Zhang***[52]	2020	5	1a, 1c
		Detection of bacterial biomass following exposure to antimicrobials using MALDI-TOF mass spectroscopy	No	Idelevich[136]	2018	4	1 b
		Detection of current through electrodes integrated within microfluidic channels containing antimicrobials plus bacterial cultures	Yes	*Fande*[137]	2023	4	1a, 1c
		Detection of bacterial metabolic activity during exposure to antimicrobials using heavy lysine labelling and MALDI-TOF mass spectroscopy	No	Dadwal[138]	2023	4	1b
		Detection of volatile organic compound spectra produced by bacteria exposed to antimicrobials using mass spectrometry	No	Dixon[139]	2022	4	1a
		Detection of catalase-positive bacterial metabolic activity during exposure to antimicrobials using catalase-inactivated luminescence	No	Wang[140]	2022	4	1b
		Detection of bacterial metabolic activity during exposure to antimicrobials using Raman spectroscopy	No	Verma[141]	2021	4	1a
		Detection of bacterial biomass following exposure to antimicrobials using MALDI-TOF mass spectroscopy	No	Idelevich[142]	2021	4	1b
		Detection of bacterial metabolic activity during exposure to antimicrobials using surface-enhanced Raman spectroscopy interpreted via machine learning algorithms	No	Thrift[143]	2020	4	1a

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Table 4 (continued) | Spectroscopy-based, laser-based, and non-optical methods for phenotypic antimicrobial susceptibility testing

Technology principles	Notable features of technology principle[a]	Detailed technology description	Quantitative MIC result	First author	Publication year	Rapid AST TRL	Phase of clinical validation
		Visualization of bacterial metabolic activity during exposure to antimicrobials within a capillary using a metabolic indicator dye and light absorbance measurements	Yes	Wang[144]	2020	4	1a, 1b
		Detection of individual bacterial metabolic activity during exposure to antimicrobials using Raman spectroscopy	Yes	Novelli-Rousseau[145]	2018	4	1a
		Detection of heat produced by bacteria exposed to antimicrobials	No	Entenza[146]	2014	4	1a, 1b

Description of methods available in published literature but not commercially available ordered according to their Rapid AST Technology Readiness Level (TRL) (definitions in Table 1), followed by date of publication. Italicized first author indicates direct-from-urine specimen capability, bolded first author indicates direct-from-positive blood culture bottle capability, and bolded plus italicized first author indicates both direct-from-urine specimen and direct-from-positive blood culture bottle capabilities. Phase of Clinical Validation also reported (definitions in Fig. 2).

[a]The net advantages and limitations of mature diagnostic platforms almost always reflect a balance of multiple elements beyond a single underlying technology that determine overall usability.

antimicrobials against *Enterobacterales* spp., as would be expected from urinary tract infections. Data on a broader diversity of drug-organism combinations are needed for clinical use. Of note, diverse technologies were employed in this group, including detection of bacterial metabolic activity during exposure to antimicrobials using deuterium labelling and stimulated Raman spectroscopy with single-cell resolution[52,53], visualization of bacterial concentration using flow cytometry and a universal fluorescent dye (without use of nucleic-acid based probes)[50], and deep-learning enabled detection of bacterial metabolic activity using the change in reflected light spectrum of a microfluidic cassette nanosurface in combination with resazurin reduction[51].

Hypothesis-free nucleic-acid based AST platforms

The capacity of metagenomic sequencing to provide hypothesis-free identification of whole-genome sequences from any putative pathogen has transformed our ability to understand their pathogenesis and epidemiology[54]. Alas, the diversity of evolving antimicrobial resistance mechanisms in the face of continuous global exposure to antimicrobials leads to an imperfect relationship between genotype and drug susceptibility phenotype. This thwarts genomic sequence-based predictions of AST phenotypes that have sufficient accuracy for patient care decisions, for most drug-organism combinations. Thus, the key parameters to understanding how promising nucleic-acid based technologies are for providing rapid automated AST that are robust across time and geography are (i) whether they offer unbiased detection of bacterial targets and (ii) their ability to determine antimicrobial susceptibility by means other than genomic sequence information alone.

We identified 14 publications describing non-commercialized technologies that employed hypothesis-free nucleic acid-based methods to detect and quantify bacteria exposed to different concentrations of antimicrobials without relying on predetermined resistance genes (Table 5). Among phenotypic nucleic-acid based tests, phenotype was characterized either by nucleic acid amplification-based methods ($n = 11$), or by quantifying physical interactions with a universal DNA or RNA probe ($n = 3$). One technology reports AST directly from positive blood culture bottles using blood spiked with bacterial isolates[55]. Seven publications reported technologies designed for AST directly from urine specimens with six reporting results from clinical urine specimens[56–62].

Finally, a number of technologies we identified claim direct-from-specimen AST for specific bug-drug combinations with an output that reports susceptibility versus resistance only. However, because their description did not report the direct measurement of MIC values, and because the interpretation of MIC values as "susceptible" or "resistant" for a given bacterial isolate to a given drug requires knowledge of the bacterial species[63], we did not consider these applicable for general clinical bacteriology laboratory work which is the focus of the present manuscript. However, such technologies may have substantial value for specific use cases such a rapid high throughput screening for infection control and prevention and are included in Tables 3 and 4.

Synthesizing the adequacy of clinical diagnostic accuracy studies of new rAST platforms

Figure 2 outlines a proposed classification for diagnostic validation studies of rapid antimicrobial susceptibility testing platforms. Phase 2 study designs are only pertinent for direct-from-specimen technologies. Conversely, all technologies should undergo some version of Phase 3 studies for regulatory submissions. The design and execution of diagnostic studies is a critical determinant of whether their findings are valid and free from bias. Best practices for diagnostic studies in general have been described in detail[18,64,65].

For Phase 2-3 studies describing novel AST technologies, the most widely used metric for concordance between two methods of MIC

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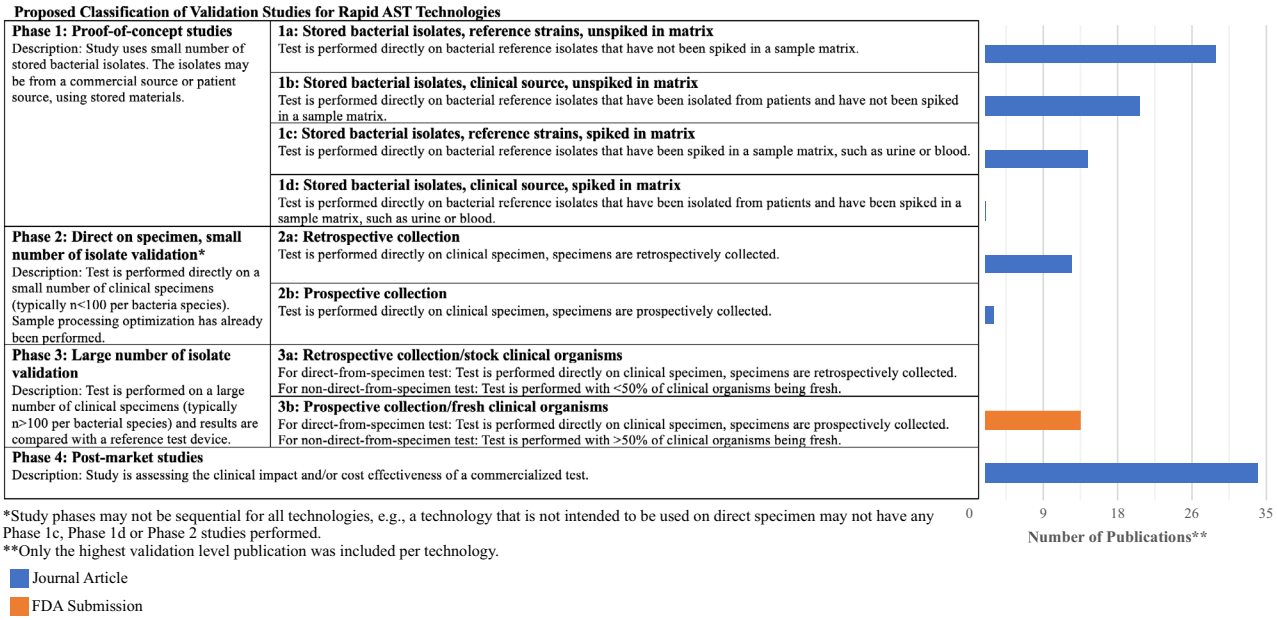

Fig. 2 | Proposed classification for diagnostic validation studies of rapid antimicrobial susceptibility testing platforms and how research studies map onto the Phase of Clinical Validation framework. This framework differs from the Technology Readiness Levels by focusing on the extent of clinical validation available from diagnostic study designs rather than stages of technological development. When a publication described the requirements for multiple phases, it was put only in the highest-level phase. All papers and FDA submissions were included in this figure. All 3b studies were from FDA submissions. One of these did not specify if the organisms were fresh or stock but referenced the FDA Class II Special Controls Document which requires >50% of organisms to be fresh, so it is assumed this criterion was met.

determination is termed essential agreement (EA) and is defined as the proportion of MIC results from an index method that are within one 2-fold dilution of that achieved with a comparator method. Compared to a reference standard such as broth microdilution, complete validation of new methods for AST determination should achieve EA of 90%, categorical agreement (CA) around susceptibility breakpoints of 90%, and "major"/"very major" errors of < 3%[66]. The precision around these parameters required by regulatory agencies typically requires testing on > 100 isolates for each bacterial species tested[67]. The origin of specimens evaluated (reference strains versus clinically derived, stored specimens versus prospectively collected ones) is a key determinant of how robust findings are likely to be in real-world use.

Thus, for commercialized rapid AST platforms with FDA authorization, approval, or a pre-market notification, performance metrics can be assumed to meet the basic standards outlined above for common bacterial species and organism-drug combinations. However, assessment of diagnostic accuracy for individual pre-commercialization technologies is complicated by the heterogeneity of the types of data that are reported. Understanding the study designs used for clinical validation (Fig. 2), and how free of bias their results are likely to be, is important for comparison between technologies in early stages of development. Performance metrics may not be directly comparable for data from different study designs or different generations of prototypes. Similarly, even within a given clinical validation phase, testing accuracy may vary significantly between bacterial species, or for a given species against different antimicrobials. For many studies we identified, diagnostic accuracy is reported for only a subset of bacterial species or antimicrobials that would be required for a regulatory submission. The Supplementary Data 1 outlines the reported performance metrics (categorical agreement, essential agreement, and errors compared to a reference standard method) as well as the combinations of bacterial species and antimicrobials tested of non-commercialized rapid phenotypic AST technologies according to phase of clinical validation, to be interpreted weighing the considerations above. Finally, the most robust data are those that are replicated by independent research groups. This is why Phase 4 studies remain important even after technologies are available commercially, as they provide real-world data on how a technology performs across use cases and its effect on clinical or health-economic outcomes.

Specific challenges facing direct-from-specimen rAST platforms

Once a specimen arrives in the laboratory, the greatest contribution to the overall turnaround-time for all currently used phenotypic AST methods—and for most of those in development—is the time required for culture amplification and colony isolation (Fig. 3). These often dwarf the time required to complete AST testing itself once bacterial colonies are isolated. Thus, rapid AST methods capable of being applied directly on clinical specimens have the potential to leapfrog incremental improvements to platforms that require isolated bacterial colonies.

However, several hurdles complicate the development of direct-from-specimen platforms. Probably the greatest technical challenge to achieving standardized MIC measurements directly-from-clinical-specimens is the lack of knowledge of bacterial concentrations (i.e. the inoculum) at the time of loading specimens into the AST device. Conventional methods work from pure isolated bacterial colonies and rely on standardized inoculum concentrations (usually 5×10^5 CFU mL^{-1}) for accurate and reproducible results[68]. Using these methods, a 100X increase in inoculum may increase the apparent MIC for some antimicrobials, while lower inoculum may artifactually decrease apparent MIC values. Both of these effects are observed especially with beta-lactam antimicrobials[69–71]. Direct-from-specimen AST platforms accordingly must be robust across a range of bacterial inoculums to be usable. Second, 6/7 direct-from-specimen AST methods we identified focused on urinary clinical specimens. This is undoubtedly because urine is a specimen matrix that is relatively free of proteins, cells and other potential inhibitory substances, compared to blood or stool for example. Moreover, clinically significant concentrations of urinary bacteria (generally considered as at least 10^5 CFUmL^{-1} from spontaneously voided first morning urine) are well

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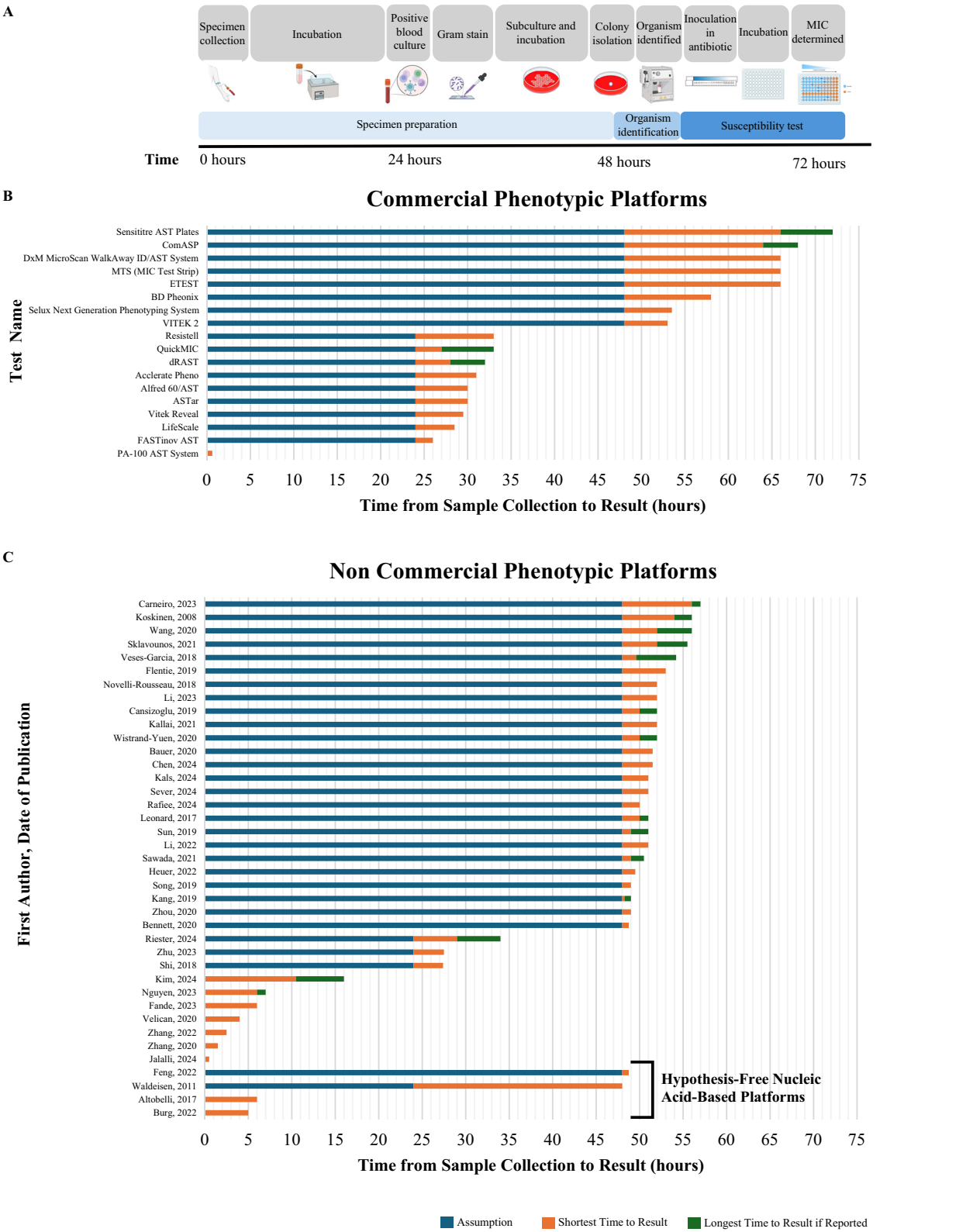

above the expected limit of detection of most detection methods. In contrast to urine, bacterial concentrations in clinical blood specimens are much lower (typically ~10^0 – 10^2 CFUmL^{-1}). Thus, while blood cultures are the cornerstone of clinical microbiology laboratories, only 1 direct-from-specimen rapid phenotypic AST platform has been validated for blood. For blood specimens, a short pre-incubation may overcome imperfect analytic sensitivity and facilitate signal detection and inoculum standardization. Alternatively, one group circumvents this by showing that bloodborne bacteria at clinically encountered concentrations can be selectively recovered and subjected to phenotypic AST[46].

Beyond direct measurement of MIC, translation of MIC values into categorical interpretation for clinicians (i.e. reporting whether bacteria A is "susceptible" or "resistant" to antimicrobial B) is based on susceptibility breakpoints that are designated for individual species of related groups of bacteria. This means that mature direct-from-

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Fig. 3 | Turnaround time of identified antimicrobial susceptibility testing (AST) technologies overlaid on a conventional AST workflow. The conventional workflow shows a timeline of the standard steps from specimen collection to final results for blood culture specimens (**A**). Cultures of urine can be assumed to require at least 24 h less than the conventional workflow shown for blood specimens. The time from specimen collection to final AST readout is shown for commercialized phenotypic platforms (**B**) and non-commercialized platforms (**C**). For commercialized phenotypic platforms, the shortest time was taken as the shortest time reported by the company and the longest time was taken from the longest time reported by the company or from a paper in our review which evaluated the platform. For non-commercialized tests, when a test reported a range of time-to-results, the shortest and longest time were recorded, otherwise only the shortest time was recorded. For tests that reported a time to result from positive blood culture, an assumption of 24 h was used to estimate the time from sample collection to the start of the test. For tests that reported a time to result from colony isolation, an assumption of 48 h was used to estimate the time from sample collection to the start of the test. When a test was performed directly from specimen collection, no assumptions of extra time were added to the total turnaround time. One assay was performed directly on blood[46], while the other direct-from-specimen tests were all performed on urine samples. 41/81 non-commercial phenotypic tests were excluded from this graph because they did not report the time from beginning the test to a minimal inhibitory concentration measurement. Graphics created with BioRender.com.

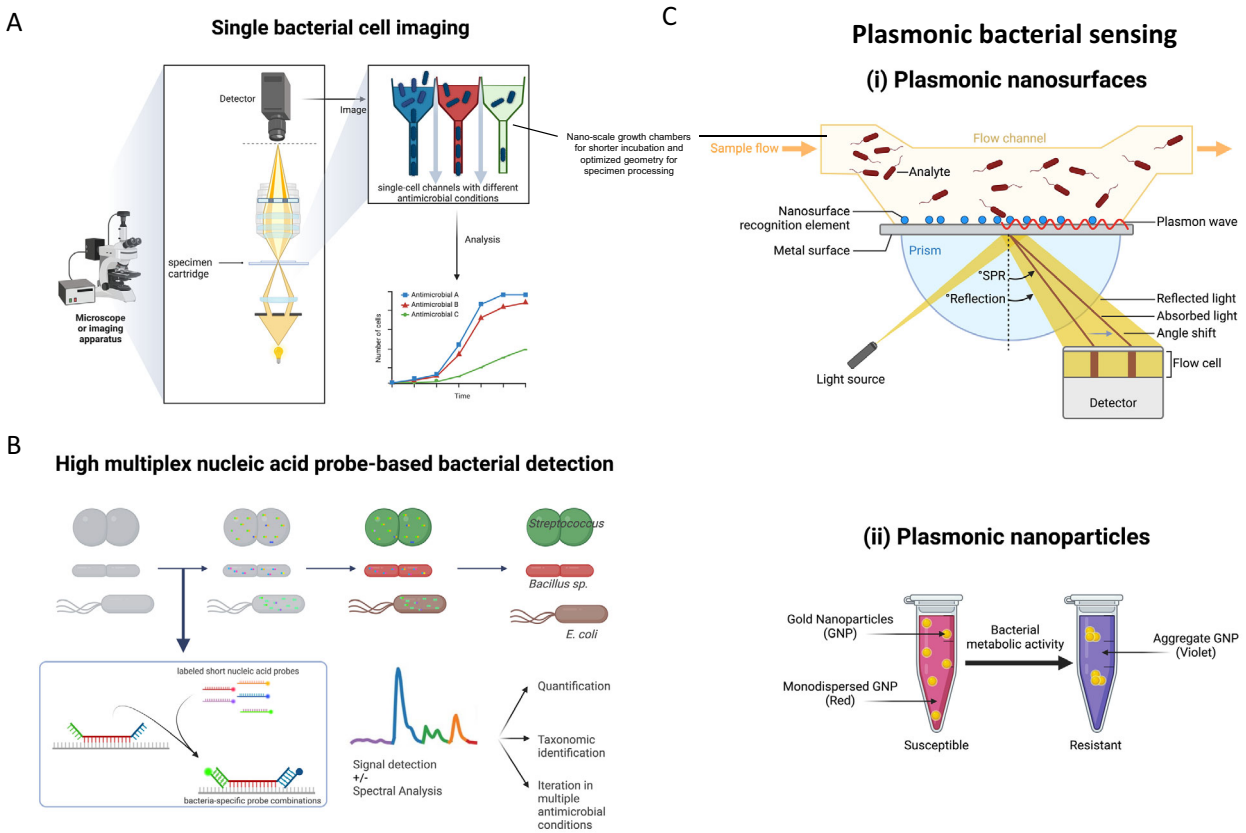

Fig. 4 | Spotlight on selected innovations underlying some of the recent advances leading to rapid phenotypic AST. Single-cell bacterial imaging (**A**) combines optical detection of bacterial growth with small reaction chambers to reduce incubation times, and optimizes geometry for specimen processing. Nanoscale growth chambers are a common feature across several rapid AST technologies. Plasmonic bacterial sensing (**B**) can enhance the speed and sensitivity of optical readouts. It can be categorized into nanosurface or nanoparticle plasmonic sensing. Nanosurface plasmonic sensing (i) incorporates nano-structured surfaces exhibiting plasmonic structural colours that enhance AST metabolic assay TAT through bright field microscopy. Alternatively, nanosurface plasmonic sensing can utilize surface plasmon resonance (SPR), where a light source illuminates the sensor under-surface through a prism using a wide beam within the range of total internal reflection. The SPR angle shifts in response to changes in the refractive index at the surface of the chip, triggered by the binding of an analyte (e.g. a biomolecule). Nanoparticle plasmonic sensing (ii) employs plasmonic nanoparticles (e.g. gold nanoparticles) for colorimetric AST detection. Changes in the configuration of the nanoparticles (monodispersed or aggregate) yield detectable colour changes (e.g. from red to violet). Different assays integrate plasmonic nanoparticle sensing where the metabolic activity of viable bacterial cells would lead to change of the nanoparticle composition (from monodispersed to aggregate or vice versa) which can be monitored by naked eye or through absorption measurements. Highly-multiplex nucleic acid probe-based bacterial detection (**C**) uses the incorporation of multiple target-specific probes into replicating bacteria and leverages spectral analysis to assign a quantification and specific identification of bacteria present. Other examples of bacterial probes include the use of isotope labels in Raman-spectroscopy-based methods. Regardless of signal detection method, deep learning is increasingly used for enhanced signal processing. Created with BioRender.com.

specimen rapid AST platforms must be combined with a method for bacterial identification if they do not provide it inherently.

Finally, several testing parameters require optimization for application of such methods to the full range of antimicrobials of interest and the spectrum of pathogenic bacteria that may be encountered in clinical use. The testing medium must be shown to support the growth of both common and fastidious organisms (such as *Haemophilus* sp. *Neisseria* sp., and *Streptococcus pneumoniae*). For antimicrobials that require non-standard concentrations of divalent cations (eg daptomycin) or other physicochemical requirements (e.g. dalbavancin)[63], dedicated AST cassettes or media are likely required.

Table 5 | Description of hypothesis-free nucleic acid-based antimicrobial susceptibility testing methods available in published literature but not commercially available ordered according to their Rapid AST Technology Readiness Level (TRL) (see Table 1 for definitions), followed by date of publication

Technology principles	Notable features of technology principle[a]	Detailed technology description	First author	Publication year	Rapid AST TRL	Phase of clinical validation
Bacterial concentration detected via electrochemical interactions	Advantages: Increased sensitivity with enzyme tags that amplify the signal and generate a current. Does not require trained personnel to interpret results. If species-specific probe used, can be combined with species identification and eliminate the need for target purification Disadvantages: Enzymes used in the redox reaction are unstable for storage, species-specific probes limit detection to 1 species and therefore challenging to make a universal assay, universal probes lose the ability for species identification	Detection of current generated by redox reaction following bacterial species-specific 16S rRNA probe binding to bacteria and brief exposure to antimicrobials	Chen[147]	2021	7	1a, 1b
		Detection of current generated by redox reaction following bacterial class-specific or universal 16S rRNA probe binding to bacteria and brief exposure to antimicrobials	*Altobelli*[56]	2017	6	2a
		Detection of current generated by redox reaction following bacterial class-specific or universal 16S rRNA probe binding to bacteria and brief exposure to antimicrobials	*Mach*[57]	2010	6	1a, 1b, 2b
Bacterial phenotype characterized by sequencing or nucleic acid amplification-based methods	Advantages: Can be combined with species identification, if sequences are specific enough then can eliminate the need for target purification. Disadvantages: Species-specific probes limit detection to 1 species and therefore challenging to make a universal assay, universal probes lose the ability for species identification. Time taken for nucleic acid amplification means that results may not be real-time. Nucleic acid amplification may require expensive/specialized reagents and equipment.	Visualization of bacterial replication during exposure to antimicrobials using fluorescence with single-cell resolution and species-specific DNA probe used to attach fluorescent dye; magnetic bead component allows physical separation from sample matrix prior to imaging	*Burg*[58]	2022	6	1a, 2a, 3a
		Quantitative pyrosequencing of bacterial DNA following brief exposure to antimicrobials and labelling of aliquots exposed to each antimicrobial to allow multiplexing of pyrosequencing reaction	*Feng*[59]	2022	6	1a, 2a
		Quantitative digital LAMP of bacterial DNA following brief exposure to antimicrobials	*Schoepp*[60]	2017	6	2a
		Quantitative LAMP of bacterial class-specific 23S rRNA following brief exposure to antimicrobials	*Schoepp*[61]	2020	5	1b, 1 d, 2a
		Quantitative digital LAMP of bacterial DNA following brief exposure to antimicrobials	Schoepp[148]	2016	5	1b, 1 d
		Quantitative real-time PCR of bacterial 16S rRNA following brief exposure to antimicrobials	**Waldeisen**[55]	2011	5	1c
		Quantitative digital PCR of bacterial DNA following brief exposure to antimicrobials	Tjandra[149]	2023	4	1a
		Quantitative digital PCR of bacterial DNA following brief exposure to antimicrobials	*Athamanolap*[62]	2019	4	1a, 1c
		Quantitative digital PCR of bacterial 16S rRNA within microfluidic device following brief exposure to antimicrobials	Athamanolap[150]	2018	4	1a
		Quantitative PCR of bacterial DNA (16S or rpoB genes) following brief exposure to antimicrobials	Rolain[151]	2004	4	1a
		Digital quantification of rolling circle amplification of probes targeting 16 rRNA region following brief exposure to antimicrobials	Mezger[152]	2015	6	1c, 2a

Italicized first author indicates direct-from-urine specimen capability and bolded first author indicates direct-from-positive blood culture bottle capability. Phase of Clinical Validation also reported (see Fig. 2 for definitions).

[a]The net advantages and limitations of mature diagnostic platforms almost always reflect a balance of multiple elements beyond a single underlying technology that determine overall usability.

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Commercialization is only the first step: obstacles to implementing diagnostics where they are needed

Value-chains for global health diagnostics are complex and fragmented[72]. Many technical and regulatory challenges must be overcome between proof-of-concept and commercialization of tests with acceptable accuracy, costs, and complexity. In addition, pitfalls especially pertinent to low-resource settings include inadequate evaluation in settings of intended use and weak end-user involvement during development stages. Post-commercialization, a second "valley of death" threatens the successful scale-up of useful technologies[73]. Hazards leading to product failures in the roll-out phase include lack of focus on demand generation, uncertain cost-effectiveness, and weak engagement of country decision-makers and stakeholders (e.g. professional societies and clinical guideline bodies; legislative bodies shaping health priorities; the financial sector for adequate prioritization of foreign exchange allocations). Several guides have been created to navigate this process[74], and it is hoped that new initiatives will bridge the current gaps facing the successful dissemination of critical AMR diagnostics to sub-Saharan Africa and Asia where needs are greatest[75,76].

Economic models that support successful post-commercialisation scale-up of diagnostic technologies in low resource settings are a multisectoral challenge. Strategies to face it have included supranational pooled procurement mechanisms such as those provided by the Global Fund[77], product-development partnerships that support the development and commercialization of priority products throughout their lifecycle[78], and the provision target product profiles specifying device parameters and ideal costs for products targeting LMIC markets[77]. This has been done for blood culture systems, for example[26]. For the new technologies identified in this review, health-economic studies evaluating different implementation models, evolving technology paradigms, dynamic health ecology, and the value placed on prevention will be required for the optimal deployment of new diagnostic technologies aimed at combatting AMR.

New technologies are looked to in the hopes that they will circumvent some of the obstacles to the implementation of conventional bacteriology in low resource settings. However, the imperative of rapidly implementing some type of phenotypic bacteriology AST in LRS should supersede emphasis on any single technological approach – whether new or conventional.

Conclusion

We synthesized the current AST pipeline and highlight necessary milestones yet to be achieved for individual technologies. Overall, a few recent advances promise rapid and accurate phenotypic AST directly from urine specimens. For blood specimens, only one non-commerical technology offers phenotypic AST without prior culture-amplification. However, robust platforms working directly from positive blood culture bottles are themselves a transformative advance. As expected, there is a relative lack of phase 2-3 clinical studies among the non-commercialized technologies we identified, possibly owing to obstacles facing diagnostic technologies on the path to commercialization[79] or the desire not to publish results prior to regulatory submissions. We did not systematically assess the suitability of technologies for low resource settings, but several of the rapid phenotypic AST technologies we identified have the potential to bridge a critical diagnostic gap in settings where the AMR crisis is most acute. With adequate prioritization and incentives from the global AMR community, it is plausible that such platforms could be deployed in the next decade.

Data availability

The data supporting the results of this work are available within the paper and its Supplementary Data 1. The raw datasets generated and analysed during the study are available from the corresponding author upon reasonable request.

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G.R., K.H. and C.P.Y. conceived the review and wrote the first draft. G.R. and K.H. performed the literature search and performed data extraction. M.P.C., J.P., and D.N. contributed to elaboration of the TRL and clinical validation frameworks. S.M. and T.A. assisted with technology descriptions. R.C., C.C., and the authors above contributed to the iterations of the search strategy, data presentation, figure design, and intellectual content of the manuscript.

Competing interests

The authors declare the following competing interests. S.M., D.N., and C.P.Y. co-supervised the work in the publication describing the PhenEXA system. C.P.Y. reports being on an Independent Data Monitoring Committees (IDMC) for Medicago Inc. and InventVacc Biologicals Inc., unrelated to the submitted work. M.P.C. reports personal fees from GEn1E Lifesciences (as a member of the scientific advisory board), personal fees from nplex biosciences (as a member of the scientific advisory board), outside the submitted work. He is the co-founder of Kanvas Biosciences and owns equity in the company. In addition, M.P.C. has a patent Methods for detecting tissue damage, graft versus host disease, and infections using cell-free DNA profiling pending, and a patent Methods for assessing the severity and progression of SARS-CoV-2 infections using cell-free DNA pending. J.P. reports grants from MedImmune, Merck; personal fees from Astra-Zeneca and Merck, all outside the submitted work. All other Authors declare no competing interests.

Additional information

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