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CD Chavda
M.V.Sc. Scholar, Department of Veterinary Microbiology, Kamdhenu University, Junagadh, Gujarat, India

JB Kathiria
Faculty of Veterinary Public Health and Epidemiology, KU, Junagadh, Gujarat, India

BJ Trangadiya
Faculty of Veterinary Pathology, KU, Junagadh, Gujarat, India

DB Barad
Faculty of Veterinary Microbiology, KU, Junagadh, Gujarat, India

BB Javia
Faculty of Veterinary Microbiology, KU, Junagadh, Gujarat, India

SN Ghodasara
Faculty of Veterinary Microbiology, KU, Junagadh, Gujarat, India

Corresponding Author:
CD Chavda
M.V.Sc. Scholar, Department of Veterinary Microbiology, Kamdhenu University, Junagadh, Gujarat, India

Antimicrobial resistance: Modern approaches for its detection

CD Chavda, JB Kathiria, BJ Trangadiya, DB Barad, BB Javia and SN Ghodasara

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Abstract
Antimicrobial resistance (AMR) poses a profound global public health crisis, fueled by overuse and misuse of antibiotics in human medicine and agriculture, resulting in the rapid dissemination of resistant pathogens and resistance determinants with devastating consequences for mortality, healthcare delivery, and economic stability. Resistance has shadowed antibiotic deployment since the earliest clinical applications, emerging swiftly after the introduction of landmark drugs during the Golden Age of discovery and continuing to challenge even last-resort agents like carbapenems and vancomycin. Bacteria employ diverse strategies to evade antimicrobials, including impaired drug permeation, targ*et al*teration, enzymatic inactivation, and active efflux, with Gram-negative species particularly adept at combining multiple mechanisms. This comprehensive review evaluates established and emerging technologies for detecting AMR and performing antimicrobial susceptibility testing, encompassing traditional phenotypic approaches (disk diffusion, broth microdilution, and automated systems), molecular techniques (PCR variants, isothermal amplification, and microarrays), advanced spectroscopic methods (MALDI-TOF MS and FTIR), sequencing-based strategies (whole-genome and metagenomic sequencing), and innovative microfluidic lab-on-a-chip platforms that enable rapid genotypic and phenotypic analysis. While these advancements promise faster, more precise diagnostics to support targeted therapy and stewardship efforts, persistent barriers such as cost, standardization, accessibility in resource-limited settings, and integration of sample preparation steps must be addressed through multidisciplinary collaboration to curb the escalating AMR threat effectively.

Keywords: Antimicrobial resistance (AMR), antibiotic misuse, resistance mechanisms, rapid diagnostics, whole-genome sequencing, microfluidic technologies

1. Introduction
Antimicrobial Resistance (AMR) has become one of the most significant health challenges of our time. Antibiotic resistance occurs as a natural evolutionary process in bacteria, but can be accelerated by many factors [1,2]. More specifically, the excessive and inadequate use of antibiotics in both humans and animals leads to the widespread spread of resistant bacteria and their antimicrobial-resistant genes (ARGs) [3]. AMR severely affects humans, healthcare systems, farm animals, agriculture, environmental health, and, consequently, national economies [4]. AMR is a challenging threat undermining key features of current medical care at enormous costs in terms of patient mortality and morbidity, but also in terms of patient treatment expenses [5]. Modern, mainstream antibiotic therapeutic strategies are responsible for their regression by actively selecting resistant strains, compelling the need to support the continuous discovery of new antibiotics to remain ahead of the AMR challenge [6]. Therefore, it is urgent to prolong the lifespan of current antibiotics while research and development of new-generation antibiotics take their course. In addition, it is important to implement efficient control measures for antibiotic use to slow down the need for continuous discovery of new antibiotics [2].
If no effective measures are taken, the costs linked to the fast-growing problem of antimicrobial resistance (AMR) are expected to increase substantially [7]. The scarcity of effective antimicrobial agents is causing routine infections to become potentially fatal, thereby

increasing the risk of treatments like chemotherapy and surgeries becoming life-threatening due to ordinary infections. Controlling the overuse and inappropriate use of antimicrobials is critical to slowing the emergence and spread of antimicrobial resistance (AMR). Recent data indicate that more than 33,000 people die each year in the European Union from infections caused by antibiotic-resistant bacteria [8]. The economic burden of AMR in the EU is considerable, reaching approximately 1.5 billion euros annually when combining direct healthcare costs and productivity losses [7]. Globally, antibiotic resistance is responsible for over half a million deaths every year, with children under five years of age representing more than 40% of these fatalities.

For infectious diseases to be effectively treated, pathogens must be promptly identified. Even with major advances in medical technology, it can still take several days to identify and characterize microbial infections [9]. This strategy may increase the ongoing antimicrobial resistance (AMR) crisis in addition to having detrimental consequences on the patient's health (such as disturbance of the microbiota). As a result, quick, dependable, reasonably priced, and economical diagnostic platforms for AMR detection are desperately needed. Implementing these platforms will substantially decrease the turnaround time for determining antibiotic susceptibility, thereby facilitating the selection of more targeted treatments [10]. Diagnostic tests are a critical component in strategies to combat antimicrobial resistance (AMR). Rapid diagnostic tests (RDTs) for infectious diseases play a vital role in antimicrobial stewardship initiatives. These tests can reduce mortality rates, less hospital stays, and reduce health expenses. Moreover, such diagnostics are highly cost-effective, significantly cutting costs while also decreasing the reliance on antibiotics. [11].

2. Historical Timeline of Major Antibiotic Discoveries and Antibiotic Resistance

Paul Ehrlich created the synthetic prodrugs salvarsan and neosalvarsan in 1910 to cure syphilis brought on by *Treponema pallidum*, marking the start of the modern era of antibiotics. Prontosil, a sulfonamide prodrug found by bacteriologist Gerhard Domagk, later mainly replaced salvarsan. American microbiologist and biochemist Selman Waksman conducted groundbreaking research on soil bacteria and their ability to produce antimicrobial chemicals in the 1930s. He defined an antibiotic as "a substance produced by one microbe to eliminate other microbes". He isolated many antibiotics from soil-dwelling filamentous actinomycetes, including streptomycin, a crucial therapy for tuberculosis.

In 1928, Scottish physician and microbiologist Sir Alexander Fleming discovered penicillin from the mold *Penicillium rubens*, launching the rapid expansion of antibiotic discovery that peaked in the mid-1950s. The era spanning the 1940s to the 1960s is widely known as the “Golden Age” of antibiotic Development, during which the majority of antibiotics still used today were first identified. Since then, the rate of new antibiotic discoveries has steadily declined, while antibiotic-resistant bacteria have continued to emerge. Resistance to antibiotics has been documented almost from the very beginning of their clinical use [12]. Even before penicillin was widely deployed as a therapeutic agent in 1940, the first penicillin-resistant *Staphylococcus* strain had already been observed. Methicillin, the first semisynthetic penicillin designed to withstand penicillinase, was introduced in 1959, yet methicillin-resistant Staphylococcus aureus (MRSA) was reported just one year later, in 1960 [13]. Vancomycin, a glycopeptide antibiotic, was approved in 1958, became a vital last-resort option for methicillin-resistant Staphylococcal infections. Nevertheless, resistance appeared relatively quickly: vancomycin-resistant coagulase-negative staphylococci (CoNS) were detected by 1979, vancomycin-resistant *Enterococcus* (VRE) emerged in 1989, vancomycin-intermediate *S. aureus* (VISA) was identified in 1997, and fully vancomycin-resistant *S. aureus* (VRSA) was documented in 2002 [14]. Cephalosporins, a class of β-lactam antibiotics, were first discovered in 1945 and entered clinical practice in 1964 as an important alternative for treating penicillin-resistant infections. Over the decades, five generations of cephalosporins have been developed, with the fifth generation now in clinical use. Later generations demonstrated strong activity, especially against gram-negative bacteria that produce extended-spectrum beta-lactamases (ESBLs). Despite this, substantial resistance has developed to all generations through the fourth. Tetracycline, another major antibiotic class, was discovered in 1950 and quickly became widely used for a broad range of infections, including gastrointestinal illnesses. However, resistance emerged rapidly: tetracycline-resistant strains of *Shigella* were already reported by 1959, less than ten years after its introduction. Levofloxacin, a 3rd-generation fluoroquinolone introduced in 1996, faced resistance from *Pneumococcus* strains reported in the same year [15]. Carbapenems, another β-lactam class introduced in the 1980s, were initially held in reserve as a vital last-line therapy against multidrug-resistant *Enterobacterales*, especially those no longer responding to cephalosporins. However, intensified clinical use throughout the 1990s and 2000s led to the global emergence of carbapenem-resistant *Enterobacterales* (CRE) from 2006 onward [16]. The history of antibiotic development shows that novel antibiotic classes were introduced by pharmaceutical companies primarily during the two decades from 1960 to 1980, followed by a significant slowdown in the pace of discovery until recent times [17]. The timeline of major antibiotic discoveries and the emergence of resistance is illustrated below (Figure).

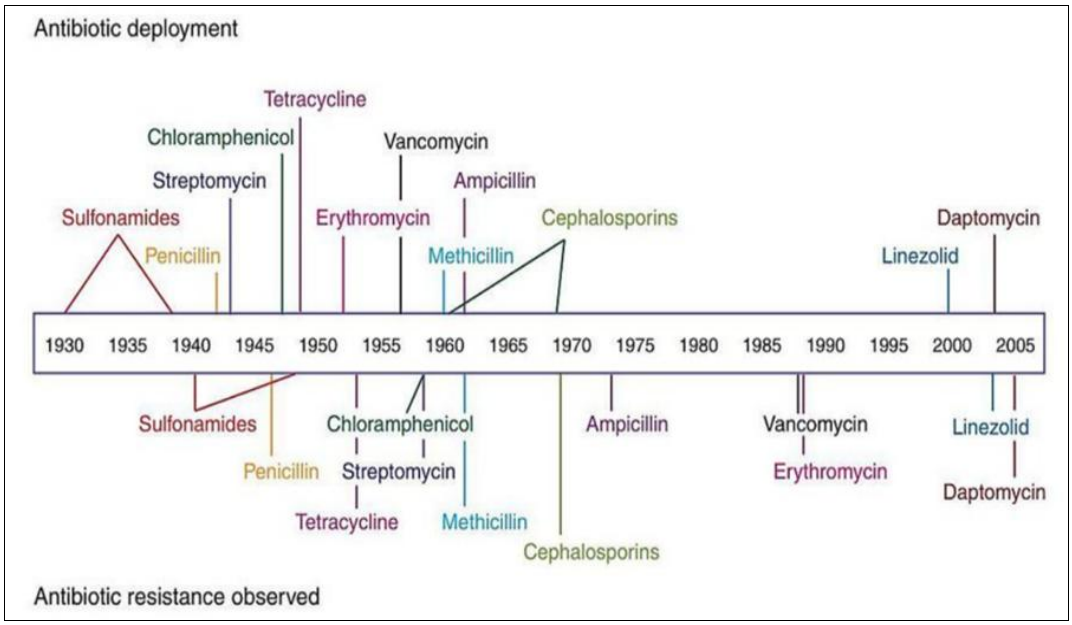

Fig: Timeline of the development of drugs and their resistance

This review seeks to provide an overview of current and developing methods and technologies aimed at the rapid detection of AMR. It also outlines the primary benefits and drawbacks of these approaches.

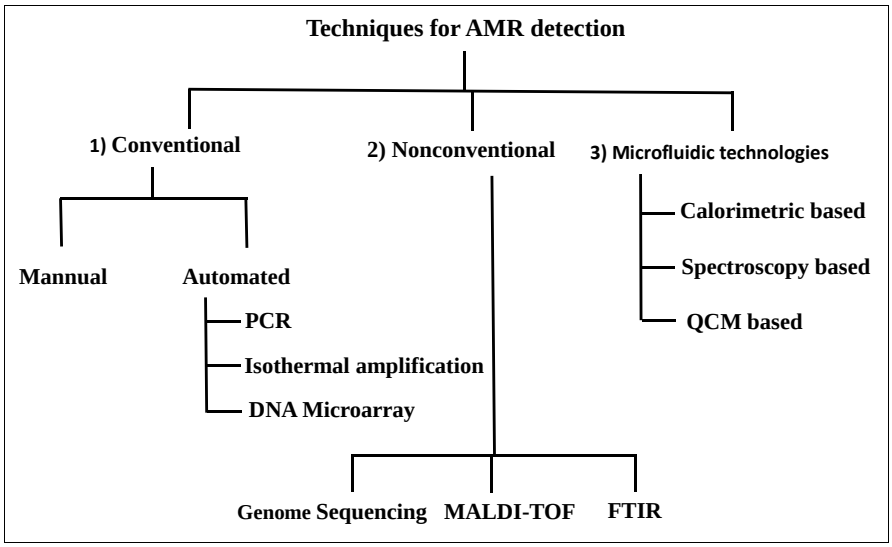

Chart: Methods and technologies analyzed in the present review.

Established approaches, including phenotypic and molecular techniques, as well as more advanced tools such as whole-genome sequencing (WGS), metagenomic whole-genome sequencing (WGM), MALDI-TOF mass spectrometry, and infrared (IR) spectroscopy, are thoroughly assessed. Special attention is devoted to emerging technologies particularly microfluidics and lab-on-a-chip systems that hold considerable potential for rapid and accurate detection of antimicrobial resistance (AMR). The discussion also includes an overview of currently available commercial platforms for antimicrobial susceptibility testing (AST). A summary chart accompanying the review outlines the key methods and technologies covered.

3. Mechanisms of Action of Antimicrobial Drugs

Every antimicrobial drug employed by clinicians operates through a specific mechanism of action (MOA) that targets microorganisms. The accompanying table lists various antibiotics alongside their respective mechanisms of action.

Table 1: Mechanism of Action (MOA) of Antimicrobial Agents

MOA	Antimicrobials
Inhibit cell wall synthesis	β-Lactams
	Carbapenems
	Cephalosporins
	Monobactams
	Penicillins
	Glycopeptides
Depolarize cell membrane	Lipopeptides

Inhibit protein synthesis	Bind to ribosomal 30S subunit
	Aminoglycosides
	Tetracyclines
	Bind to ribosomal 50S subunit
	Chloramphenicol
	Lincosamides
	Macrolides
Inhibit nucleic acid synthesis	Quinolones
	Fluoroquinolones
Inhibit metabolic pathways	Sulfonamides
	Trimethoprim

3.1 AMR Mechanisms

Antibiotic resistance can be broadly classified into two main types: intrinsic (natural) resistance and acquired resistance. Intrinsic resistance may be constitutive, meaning the resistance mechanisms are always expressed as a natural characteristic of the bacterial species, or inducible, where resistance genes are already present in the genome but are expressed at clinically significant levels only after the bacterium is exposed to an antibiotic [18]. In contrast, acquired resistance occurs when bacteria that were previously susceptible gain new resistance determinants through the uptake of external genetic material via mechanisms such as transformation, conjugation, or transposition [19], or mutations in its chromosomal DNA. The mechanisms underlying antimicrobial resistance (AMR) can be grouped into four primary categories: (1) Reduced drug uptake (limiting the entry of the antibiotic into the bacterial cell); (2) Modification of the drug target (altering the site where the antibiotic normally binds); (3) Inactivation of the drug (through enzymatic degradation or chemical modification); and (4) Active efflux (pumping the antibiotic out of the cell) [20].

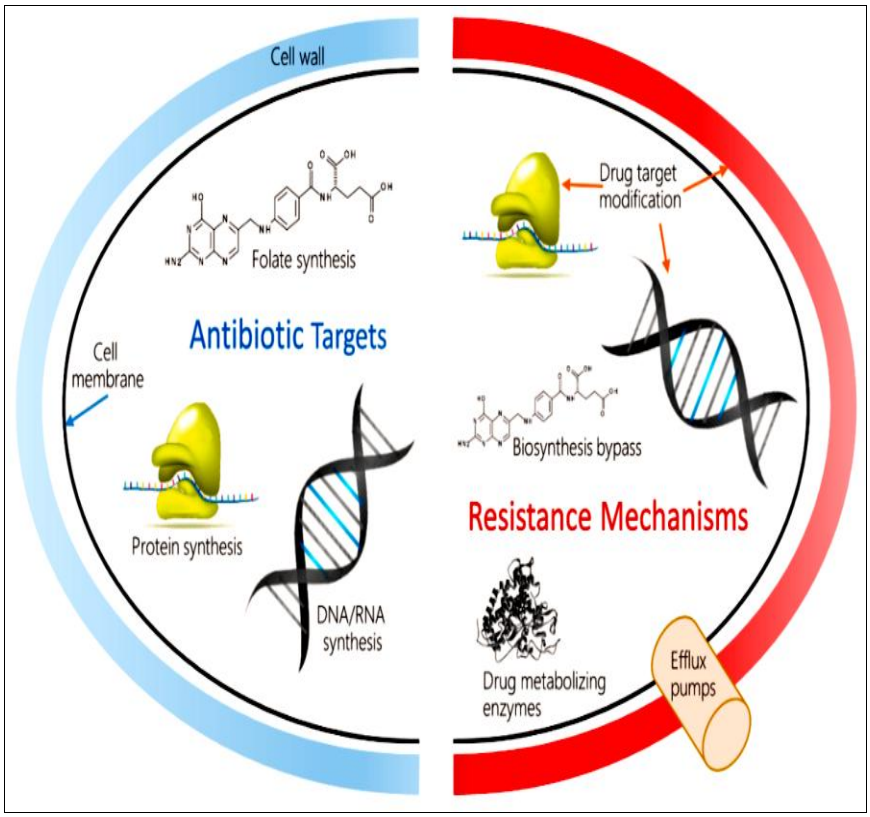

Fig: MOA of antimicrobial resistance [21]

Gram-negative bacteria can exploit all four major AMR mechanisms, while Gram-positive bacteria rarely rely on reduced drug uptake or efflux pumps due to the lack of an outer membrane. The lipopolysaccharide-rich outer membrane in Gram-negatives acts as a strong permeability barrier, conferring intrinsic resistance to many antibiotics, including large glycopeptides like vancomycin. Hydrophilic antibiotics (β-lactams, tetracyclines, some fluoroquinolones) are especially affected by reduced porin expression or porin mutations [22]. Downregulation or loss of porins is a major non-enzymatic cause of carbapenem resistance in *Enterobacterales*, *Acinetobacter*, and *Pseudomonas* [23]. In enterococci, low porin activity or replacement with less selective channels contributes to natural aminoglycoside resistance. Biofilms further protect bacteria by forming a matrix of polysaccharides, proteins, and extracellular DNA that impedes antibiotic penetration [24,25].

3.1.1 Drug Efflux

Efflux pumps actively remove many antibiotics from bacterial cells and play a major role in the intrinsic resistance of Gram-negative species. These transporters are present in nearly all bacteria and belong to five major families: ABC, SMR, MATE, RND, and MFS, distinguished by structure and energy source [18]. Only the RND family forms tripartite complexes that span both membranes and expel drugs directly

into the external environment, whereas the other four families are single-component pumps limited to the inner membrane [26]. Classic examples include Tet proteins (MFS family) that export tetracyclines using proton-motive force. Clinically important RND systems, such as MexAB-OprM in Pseudomonas aeruginosa and AcrAB-TolC in *Enterobacterales*, confer multidrug resistance by pumping out tetracyclines and other classes [27]. Macrolide resistance is frequently driven by efflux; the widely studied mef genes encode MFS-type pumps specific for 14- and 15-membered macrolides. Additionally, the ABC-family pump MacB forms the tripartite MacAB-TolC complex to expel macrolides from the cell [26]. Overexpression of these efflux systems remains a key driver of high-level, broad-spectrum resistance in many pathogens.

3.1.2 Drug inactivation

Bacteria render antibiotics ineffective primarily through two strategies: enzymatic degradation of the drug molecule or chemical modification (e.g., acetylation, phosphorylation, or adenylation) of its structure [22].

3.1.3 Chemical modification of the drug

Bacteria evade antibiotics by producing enzymes that attach chemical groups, blocking the drug from reaching its target. The most common modifications are phosphorylation, acetylation, and adenylation [28]. Acetylation predominantly inactivates aminoglycosides, chloramphenicol, streptogramins, and some fluoroquinolones, while aminoglycosides are also targeted by adenylation and phosphorylation. These aminoglycoside-modifying enzymes (AMEs) covalently alter hydroxyl or amino groups, abolishing drug activity a classic example of resistance via chemical modification [26].

3.1.4 Destroying the drug

B-Lactam antibiotics (e.g., penicillins and cephalosporins) owe their activity to a characteristic four-membered β-lactam ring. The dominant resistance mechanism is production of β-lactamases, which hydrolyze this ring and prevent binding to penicillin-binding proteins (PBPs) [29]. Target modification is another widespread resistance strategy. Bacteria can alter the structure or expression level of PBPs, reducing β-lactam affinity; a classic case is the mecA gene in S. aureus, which encodes a low-affinity PBP2a [30]. Similarly, erm genes methylate 23S rRNA, blocking macrolides, lincosamides, and streptogramins from their ribosomal target. Resistance to fluoroquinolones commonly arises from mutations in DNA gyrase or topoisomerase IV that impair drug binding [31].

4. Techniques for AMR detection

4.1 Conventional Methods for AMR Detection

Although rapid modern technologies now exist for AMR detection, traditional culture-based and molecular methods remain widely used. Recently, microscopy- and spectrometry-based approaches have also been added to diagnostic toolkits.

4.1.1 Manual and Automated methods

4.1.1.1 Phenotypic Methods

Phenotypic culture-based methods for detecting antibiotic resistance are classified as manual (disk diffusion, broth microdilution, agar dilution, E-test) or automated (e.g., VITEK®2, Sensititre™, Alfred 60AST). Automated platforms use pre-dosed cartridges or micro-titre plates (such as Sensititre panels) containing dried antibiotics at graduated concentrations, enabling real-time growth monitoring and precise MIC determination. These systems deliver both qualitative (susceptible/intermediate/resistant) and quantitative (MIC) results; for example, broth microdilution and E-test provide the lowest concentration inhibiting visible growth, while disk diffusion measures inhibition zone diameter [32]. The E-test gradient strip is especially useful for fastidious pathogens like Campylobacter [33]. Result interpretation follows annually updated breakpoints from EUCAST and CLSI, though differences exist e.g., EUCAST uses a stricter amikacin breakpoint of ≤8 mg/L for E. coli, whereas CLSI uses ≤16 mg/L. Despite newer technologies, these conventional methods remain the gold standard for routine antimicrobial susceptibility testing worldwide.

4.1.2 Genotypic Methods

4.1.2.1 Molecular-Based Methods

Molecular-based assays for detecting antimicrobial resistance genes (ARGs) provide several benefits compared to phenotypic methods, including the ability to target multiple genes simultaneously and offer precise identification and characterization of AMR genes. For certain taxonomic groups where susceptibility breakpoints are not defined, molecular methods serve as a viable alternative. Additionally, these assays do not require isolate purification, as they can process non-purified polymicrobial samples. They also enable rapid adaptation to newly emerging resistance factors [34]. Molecular-based assays for detecting antimicrobial resistance (AMR) have certain limitations. They cannot determine the minimum inhibitory concentration (MIC). Additionally, these methods may miss some antimicrobial resistance genes (ARGs) due to limitations in sensitivity and coverage, as they only detect known resistance genes and not newly emerged ones. The wide variety of AMR-related genes also complicates assay development due to high costs, making it challenging to compete with phenotypic assays in some cases. Despite these drawbacks, advancements in molecular techniques are increasingly establishing their role in routine diagnostics [35]. These methods, which detect ARGs and their expression, leverage progress in nucleic acid amplification and hybridization technologies [36]. Molecular approaches enable rapid and sensitive ARG detection, where ARGs encode bacterial mechanisms to survive and proliferate in the presence of antibiotics. Previously, researchers targeted only a limited set of ARGs, but with the reduced costs of next-generation sequencing (NGS) and the growth of bacterial whole-genome sequencing (WGS), the range of ARG targets in databases has significantly expanded [37]. The following sections will explore nucleic acid amplification techniques, including polymerase chain reaction (PCR), isothermal methods, and DNA microarrays.

4.1.2.2 PCR-Based Methods

Polymerase chain reaction (PCR) is the most widely utilized nucleic acid amplification method for identifying antimicrobial resistance genes (ARGs) [38,39]. More recently, real-time [40], quantitative [41], Digital [42] and multiplex PCR assays, have significantly enhanced the clinical adoption of genetic testing.

Developments in next-generation sequencing (NGS) and whole-genome sequencing (WGS) have expanded the availability of ARG targets, enabling high-throughput quantitative PCR (HT-qPCR). This method is rapid, cost-effective, and facilitates simultaneous analysis of numerous ARGs [37,43]. HT-qPCR has been applied in various studies to

examine ARGs from diverse sample types, such as park soil bacteria [37]. HT-qPCR has been applied in various studies to examine ARGs from diverse sample types, such as park soil bacteria [43], Additionally, a novel high-throughput screening approach using HT-qPCR tested the antimicrobial susceptibility of *Orientia tsutsugamushi* clinical isolates against three antibiotics, screening 48 isolates simultaneously [44]. Xu *et al*. showed that chemically synthesized double-stranded (ds) DNA can serve as a reliable qPCR standard for ARGs, matching the sensitivity and performance of natural DNA across sample types like animal feces, soil, and surface water.

A multiplex real-time PCR assay was used to characterize antimicrobial resistance in *Neisseria gonorrhoeae*, detecting resistance to antibiotics such as ciprofloxacin, ceftriaxone, cefixime, azithromycin, and spectinomycin. Although this method accurately identified resistance-conferring mutations for gonorrhea treatment, its low sensitivity limits its use for direct diagnostic testing in clinical samples. However, it remains effective for screening AMR in gonococcal isolates, offering faster results than traditional culture-based methods [45]. Similarly, singleplex and multiplex real-time PCR assays for methicillin-resistant *Staphylococcus aureus* (MRSA) in pediatric samples demonstrated speed, reliability, and the ability to distinguish between MRSA and methicillin-susceptible *S. aureus* (MSSA) [43]. Two decades ago, ligation-mediated PCR (LM PCR) combined with a low denaturation temperature technique was developed, producing specific DNA melting-profile patterns for fungal and bacterial isolates. This approach is effective for strain characterization and differentiation [46]. It has been applied for epidemiological typing of pathogens, including extended-spectrum beta-lactamase-producing *Escherichia coli* [47] It has been applied for epidemiological typing of pathogens, including extended-spectrum beta-lactamase-producing *Escherichia coli* [47].

4.1.2.3 Isothermal Amplification Methods

A major advance in molecular diagnostics is isothermal DNA amplification, which eliminates the need for thermal cycling used in conventional PCR. Several isothermal nucleic acid amplification techniques have been developed, including strand displacement amplification (SDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and helicase-dependent amplification (HDA) [48]. These methods have paved the way for rapid, next-generation molecular diagnostic assays [49].

Isothermal DNA amplification offers key advantages over traditional PCR methods by eliminating the need for thermocycling, resulting in lower power consumption and faster analysis times. Instead of requiring thermocyclers, simple equipment like a water bath or hotplate can maintain the necessary temperature [50]. Unlike PCR, isothermal amplification is quicker and more sensitive, as it relies on continuous amplification rather than discrete thermal cycles, producing detectable amplicons in under 10 minutes [51]. Certain isothermal methods, such as loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), and helicase-dependent amplification (HDA), do not require template denaturation and, in the case of LAMP and HDA, are tolerant to biological components [52]. Additionally, despite complex primer designs in some methods like LAMP, isothermal techniques often provide greater specificity than PCR. A recent assessment of isothermal methods based on simplicity, sensitivity, cost, and reproducibility highlighted LAMP and recombinase polymerase amplification (RPA) as highly suitable for point-of-need (PON) diagnostics in low-resource settings, as they involve a single-step incubation at a constant temperature and require minimal DNA template [50]. Isothermal methods are also well-suited for microfluidic platforms due to these advantages. Furthermore, LAMP amplicons can be detected visually through turbidity or color changes. However, isothermal methods have limitations. Multiplexing is less effective due to the increased complexity of experimental design [53]. Additionally, some methods, such as LAMP, require multiple primers (4-6) or involve complex reaction mechanisms, while others, like nucleic acid sequence-based amplification (NASBA), require several enzymatic steps [52].

Over the past two decades, substantial advancements in engineering, reagent development, and software have driven the commercialization of *in vitro* diagnostic (IVD) products utilizing PCR and isothermal nucleic acid amplification technologies (NAAT) [54]. The automation and integration of processes such as nucleic acid extraction, purification, amplification, and detection, combined with advanced data analysis software, have enabled the development of fully integrated and automated platforms (discussed in later sections of this review) that deliver precise results [55].

4.1.2.4 DNA Microarrays

A DNA microarray is a technique used to evaluate bacterial genomic diversity by detecting the presence or absence of genes in a target organism compared to a reference strain or genome. Originally, DNA microarrays utilized glass slides spotted with numerous specific DNA probes based on reference genes from a fully sequenced, characterized strain. Comparative genomic hybridizations were conducted, followed by analysis of the hybridization outcomes. However, the reliance on glass slides and fluorescent dyes made the process expensive and time-intensive. Over the past two decades, significant advancements in DNA microarray technology have been made [56]. A rapid and straightforward DNA labeling method using biotinylated primers specific to linkers has been developed for disposable microarrays. A DNA microarray was created for the simultaneous detection (via multiplex asymmetric PCR amplification) of antimicrobial resistance genes (ARGs) in *Staphylococcus* clinical isolates, utilizing fluorescently labeled PCR products. More recently, Havlicek *et al*. introduced a cartridge-based, melting curve assay for detecting pyrazinamide-resistant *Mycobacterium tuberculosis*. This assay can be automated using a closed cartridge system paired with a battery-operated Alere™ q analyzer, enabling point-of-care testing in resource-constrained environments [57].

4.2 Non-Conventional AST Methods

This section highlights some of the most promising non-conventional approaches for AST. These include sequencing, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and Fourier transform infrared (FTIR) spectroscopy.

4.2.1 Genome Sequencing and Metagenomics in AMR Diagnostics

DNA sequencing methods first emerged in the mid-1970s, capable of decoding hundreds of nucleotides bases daily. The two prominent techniques at the time were the chain terminator method [58] and the chemical cleavage procedures

[59]. These methods achieved single-base resolution through polyacrylamide gel electrophoresis for each base-specific reaction. In 1995, the first complete bacterial genome, *Haemophilus influenzae* (1,830,137 bp), was sequenced using automated sequencers that employed fluorescence-based Sanger chemistry [60]. Until 2005, Sanger sequencing remained the dominant technology, offering high-quality, relatively long DNA sequences despite its low throughput. Multiple samples could be sequenced by incorporating numerous capillaries in a single instrument. The key innovation of next-generation sequencing (NGS) was multiplexing, enabling the simultaneous analysis of thousands of samples. The typical NGS workflow involves DNA extraction, fragmentation, adaptor ligation, amplification, and sequencing. However, second-generation or short-read sequencing has limitations, including copying errors, sequence-dependent biases, and information loss during template amplification. In 2005, the 454-pyrosequencing platform was introduced, which detects pyrophosphate release and light generation during nucleotide incorporation, differing from Sanger's chain termination using dideoxynucleotides [61]. Subsequently, Illumina platforms, utilizing synthesis technology with reversible terminator nucleotides labeled with fluorophores that are visualized through excitation, were developed to achieve similar goals [62].

Third-generation sequencing, introduced by Pacific Biosciences in 2011, utilizes real-time, single-molecule long-read sequencing through an optical approach combined with a zero-mode waveguide on a nanostructured device [63]. Another method, developed by Oxford Nanopore Technologies, involves DNA molecules passing through a nanopore, with an electrical signal varying according to the base traversing the pore [64]. These second- and third-generation sequencing technologies have enabled single-genome sequencing, characterization of complex microbial communities, and identification of antibiotic resistance determinants. Whole metagenome sequencing (WMS) and analysis of genetic material from patient samples allow for direct detection of antimicrobial resistance genes (ARGs) in clinical specimens without requiring prior bacterial isolation or identification. Advances in sequencing technologies have increased the availability of bacterial sequence data. Coupled with improved computational methods and decreasing costs due to competitive market dynamics, sequencing has become a cost-effective and practical tool for ARG identification, characterization, and monitoring [65]. Numerous methods, tools, and databases (Table 1) have been documented recently for detecting genetic determinants of antimicrobial resistance (AMR) from whole-genome sequencing (WGS) and WMS data [66]. These emerging technologies complement traditional culture-based methods, offering rapid and sensitive resistance detection in both cultivable and uncultivable bacteria. Detailed insights into database use for AMR detection are available in two recent reviews. Organizing sequencing data is a critical pre-processing step for ARG analysis. Short reads, such as those generated by Illumina, can be processed using assembly-based methods, where sequencing reads are assembled into contiguous fragments (contigs) and annotated by comparison with public or custom reference databases, or through read-based methods, where resistance determinants are predicted by mapping reads directly to a reference database [67].

Bioinformatic tools and databases such as RGI, CARD, ARGs-OAP (v2), ARIBA, NCBI-AMRFinder, PointFinder, ShortBRED, SEAR, KmerResistance, PATRIC, SSTAR, DeepArgs, and GROOT are utilized for detecting antimicrobial resistance genes (ARGs) from whole-genome sequencing (WGS) or whole-metagenome sequencing (WMS) data.

A significant advancement in resistome surveillance is the ability to predict antimicrobial resistance (AMR) using only genomic data. Studies, including those on foodborne pathogens, have shown a high correlation (>96%) between known mutations or ARGs and the minimum inhibitory concentration (MIC) of various antimicrobials [68]. Furthermore, increasing evidence suggests that machine learning techniques applied to genome sequencing data can predict AMR and, in some cases, the MIC of antimicrobials. While long-read sequencing platforms offer comprehensive genome coverage, they demand significant investment in equipment and laboratory expertise. These systems also require large DNA quantities (e.g., >5 µg), longer preparation times, and exhibit higher error rates compared to short-read sequencing platforms. Alternatively, nanopore-based sequencing technologies are recognized for generating high-quality libraries from long reads and producing complete bacterial genomes. Additional benefits include portability, affordability, reduced laboratory space requirements, and the ability to perform on-site sequencing. The MinION nanopore system (Oxford Nanopore, Oxford, UK), a portable (palm-sized, 100 g), real-time device for DNA and RNA sequencing, detects changes in ionic current as DNA or RNA passes through nanopores.

4.2.1.1 Pyrosequencing

In 2012, pyrosequencing was proposed as a novel, rapid method for identifying *Yersinia pestis* strains in efforts to combat bioterrorism. This approach utilized virulence genes, leading to the development of a pyrosequencing-based assay for characterizing antimicrobial resistance gene (ARG) profiles [69]. Pyrosequencing was also assessed for detecting drug-resistant *Mycobacterium tuberculosis* in clinical settings. The assay demonstrated high reliability and robustness in identifying resistance-associated mutations in *M. tuberculosis* isolates, with specificity ranging from 96% to 100% [70]. Its effectiveness was evaluated for the rapid detection of resistance to fluoroquinolones (FQs), rifampicin (RIF), kanamycin (KAN), and capreomycin (CAP) in *M. tuberculosis* clinical isolates, achieving sensitivities of 100% for RIF, 100% for FQs, 40% for CAP, and 50% for KAN, with 100% specificity. The assay was recognized as a fast and efficient method for detecting drug resistance mutations in *M. tuberculosis* clinical isolates, though it has since been surpassed by newer sequencing technologies (discussed below).

4.2.1.2 Whole Genome Sequencing

Whole-genome sequencing (WGS) was assessed for predicting antimicrobial resistance (AMR) in non-typhoidal *Salmonella* from human and food isolates, using v2 or v3 chemistry with paired-end 2x25-bp or 2x300-bp reads on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The results indicated a strong correlation between acquired resistance and known resistance determinants, supporting its utility in risk assessment related to antibiotic use in food animal production [71]. Vélez *et al*. utilized WGS to investigate the presence of antimicrobial resistance genes (ARGs) in *Streptococcus uberis* and *Streptococcus dysgalactiae* isolates from dairy cows, employing paired-end 125-bp sequencing on the Illumina HiSeq 2500 platform with

v4 chemistry. Their study also explored the relationship between genomic, epidemiological, and phenotypic AMR profiles, revealing associations between unique ARG sequences and phenotypic resistance based on minimum inhibitory concentration (MIC) data [72]. Vélez *et al*. utilized WGS to investigate the presence of antimicrobial resistance genes (ARGs) in *Streptococcus uberis* and *Streptococcus dysgalactiae* isolates from dairy cows, employing paired-end 125-bp sequencing on the Illumina HiSeq 2500 platform with v4 chemistry. Their study also explored the relationship between genomic, epidemiological, and phenotypic AMR profiles, revealing associations between unique ARG sequences and phenotypic resistance based on minimum inhibitory concentration (MIC) data [73]. These findings, supported by further studies [74], underscore WGS as a valuable tool for AMR surveillance programs. More recently, WGS was used to investigate an ongoing epidemiological shift, revealing the coexistence of antibiotic resistance and virulence factors in carbapenem-resistant *Klebsiella pneumoniae* isolates, highlighting the need to consider these findings in future genomic surveillance efforts [75].

4.2.1.3Combination of Short and Long Read WGS Sequencing

Plasmids play a key role in transferring antimicrobial resistance genes (ARGs) among bacterial isolates. However, assembling plasmids from short-read whole-genome sequencing (WGS) data is challenging. Berbers *et al*. employed a combination of short- and long-read WGS to characterize ARGs on plasmids and determine their locations. Given the growing concern over ARG dissemination, identifying their presence on mobile genetic elements like plasmids is critical. By integrating long- and short-read sequencing, the difficulties in plasmid reconstruction were addressed, enabling effective risk assessment of AMR spread [76].

4.2.1.4 Nanopore Sequencing

Nanopore sequencing has been extensively applied to viruses, yeasts, and de novo bacterial assembly [77], as well as for identifying viral pathogens, conducting metagenomics studies, and detecting antimicrobial resistance genes (ARGs) [78]. The MinION nanopore sequencer was used to determine the structure and chromosomal insertion site of a composite antibiotic resistance island in *Salmonella Typhi* [79]. It also facilitated the identification of the position and structure of AMR determinants in a multidrug-resistant (MDR) strain of enteroaggregative *Escherichia coli*. Long-read WGS analysis revealed mobile genetic elements carrying AMR determinants and showed multiple AMR determinants co-located on the same element, enhancing understanding of their transmission in MDR *E. coli* [80]. Schmidt *et al*. demonstrated that MinION could identify bacterial pathogens and acquired resistance genes directly from urine samples within 4 hours without culturing, underscoring its value for whole-metagenome sequencing (WMS)-based diagnostics to guide antimicrobial therapy [81]. The Oxford Nanopore MinION, a long-read DNA sequencing device, was utilized to detect antimicrobial resistance genes (ARGs), determine their taxonomic origins, and analyze their genetic organization and association with mobilization markers. These findings enabled targeted interventions to reduce ARG transmission risks, enhancing biosecurity in hospitals and other settings [82]. MinION sequencing also facilitated rapid identification of plasmids, virulence markers, phages, and ARGs in Shiga toxin-producing *Escherichia coli*. Additionally, it was used to identify pathogens, plasmids, and ARGs in bacterial DNA from positive blood cultures, achieving pathogen identification in 10 minutes and detecting predefined ARGs and plasmids within 1 hour using raw nanopore data. Unlike Illumina sequencing, which provides data only after the run completes, nanopore sequencing offers real-time data availability [83]. The MinION was also applied to generate and characterize whole genomes of *Streptococcus suis*, accurately predicting multilocus sequence types in 8 of 10 samples and identifying AMR profiles in all samples [84]. Ultra-long read nanopore sequencing detected ARGs in *Mannheimia haemolytica*, producing a complete genome for a non-resistant strain and a near-complete assembly for a resistant strain, with successful ARG detection using only 5,437 MinION reads [85].

Unlike phenotypic tests, which provide only antimicrobial susceptibility testing (AST) data, next-generation sequencing (NGS) elucidates the molecular basis of AMR. This information supports monitoring programs, enhances understanding of resistance acquisition, and enables characterization of novel resistance mechanisms in phenotypically resistant isolates, offering significant advantages over nucleic acid-based techniques like PCR.

4.2.2 MALDI-TOF Mass Spectrometry in AMR Diagnostics

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers an alternative to traditional genotypic or phenotypic methods for detecting antimicrobial resistance (AMR) [86]. It analyzes the cellular proteome, primarily ribosomal proteins (2-20 kD), from whole bacterial cell extracts, generating spectral fingerprints that distinguish microorganisms at the genus, species, and subspecies levels [87].

In the assay, a sample is mixed with an energy-absorbent matrix solution, which crystallizes upon drying. A laser beam ionizes the sample, producing protonated ions that are accelerated by a constant potential and separated based on their mass-to-charge (m/z) ratio. The time-of-flight (TOF) data generates a peptide mass fingerprint (PMF), a unique mass spectrum compared against a database of reference peaks for known microorganisms to identify the sample [88].

MALDI-TOF MS also detects antibiotic resistance mechanisms, such as carbapenemases, but requires standardized procedures for reproducible results [89]. It is rapid (results in minutes), accurate, user-friendly, cost-effective, and environmentally friendly. It is rapid (results in minutes), accurate, user-friendly, cost-effective, and environmentally friendly [88]. However, high costs for purchase and maintenance, large system size, and unsuitability for mixed samples (requiring prior purification, cultivation, and sample preparation) limit its use in low-resource settings or as a point-of-care (POC) platform for AMR or antimicrobial susceptibility testing (AST) [90]. Additional chemicals, like the matrix, are also needed [91]. Databases distinguishing susceptible and resistant strains are essential. Table 2 lists recent studies applying MALDI-TOF MS to AMR detection. The Bruker MALDI Biotyper (Bruker Daltonik, Bremen, Germany) and VITEK MS (bioMérieux, Marcy l'Étoile, France) are the two main commercial MALDI-TOF MS systems, with performance comparisons available in the literature [92].

4.2.3 Fourier Transform Infrared (FTIR) Spectroscopy in AMR Diagnostics

Recent advancements in optical technologies have significantly impacted biomedical and microbiology fields. Infrared (IR) spectroscopy and microscopy provide enhanced spectral and spatial resolution, enabling molecular-level biochemical analysis of microorganisms. In clinical microbiology, Fourier transform infrared (FTIR) spectroscopy, a phenotypic method, has become a powerful tool for biochemical analysis due to its ability to provide detailed molecular composition data. FTIR quantifies IR light absorption by molecules such as lipids, lipopolysaccharides, carbohydrates, proteins, and nucleic acids, generating a characteristic spectrum that reflects the sample's complete composition [93]. These spectra offer rich functional and structural insights and have been used to identify molecular changes linked to antimicrobial resistance (AMR) in prokaryotes [94].

Combining IR spectroscopy with artificial neural networks (ANNs) enabled the detection of cephalothin-susceptible uropathogenic *Escherichia coli* strains with a 95% success rate [95]. [96] Employed FTIR with an IR microscope and novel pattern-recognition tools to analyze spectra, determining *E. coli* susceptibility to ceftazidime, gentamicin, nitrofurantoin, nalidixic acid, and ofloxacin with an 85% classification accuracy for sensitive versus resistant strains.

FTIR offers advantages like reliability, speed, cost-effectiveness, and environmental friendliness for AMR studies. However, similar to MALDI-TOF MS, its high purchase and maintenance costs and large equipment size hinder implementation in low-resource settings or as a point-of-care (POC) platform for AMR or antimicrobial susceptibility testing (AST). Sample purification, cultivation, and preparation are required, and databases with spectra distinguishing susceptible and resistant strains are essential.

4.3 Microfluidics and Lab-on-a-Chip Technologies towards Rapid Diagnostics

Lab-on-a-chip (LoC) devices utilizing microfluidics are promising for applications in clinical diagnostics, food safety, and environmental monitoring [97], including the detection of antibiotic-resistant bacteria [3]. Compared to macro-scale methods, LoC technology offers rapid, high-throughput analysis, precise fluid manipulation, low cost, minimal reagent and power consumption, small sample volumes, automation, integration, compactness, and portability [98]. Microfluidic-based detection methods are divided into genotypic and phenotypic assays. Genotypic assays, such as PCR and LAMP, target genetic markers like antimicrobial resistance genes (ARGs), bypassing bacterial growth and reducing turnaround time to hours [99]. Microfluidic-based detection methods are divided into genotypic and phenotypic assays. Genotypic assays, such as PCR and LAMP, target genetic markers like antimicrobial resistance genes (ARGs), bypassing bacterial growth and reducing turnaround time to hours [50]. Combining microfluidics with isothermal DNA amplification eliminates thermal cycling, enhancing efficiency and enabling cost-effective, user-friendly diagnostic tools for food safety, clinical, and environmental applications [100]. Phenotypic assays, conversely, monitor bacterial growth in the presence of antibiotics for accurate antimicrobial susceptibility testing (AST). These assays confine bacteria in small volumes (e.g., chambers, channels, or droplets), using techniques like antibody capture on magnetic beads or membranes, or encapsulation in agarose with hydrodynamic trapping [101]. Hydrodynamic trapping, for instance, supports high-density trap arrays and easy integration but has low trapping efficiency. Antibody-based methods are costly and limited by strain-specific availability, while droplet-based methods require expensive, complex readouts. Agarose-based methods, though applicable to multi-well plates, face challenges in arraying, complicating automated detection and data analysis. Further research is needed to overcome these limitations for commercial viability. Subsequent sections will explore spectroscopy-based, colorimetric-based, pH-based, and quartz-crystal microbalance (QCM)-based approaches, as well as point-of-care, multiplexing, single-cell, and single-molecule methods.

4.3.1 Spectroscopy-Based Approaches

Surface-enhanced Raman spectroscopy (SERS) is a key biochemical fingerprinting method that accurately captures macromolecular profiles and changes in bacterial cells due to antibiotic effects [91]. It has been used to assess bacterial resistance or susceptibility to antibiotics and to study antibiotic mechanisms through whole-cell spectral fingerprints. SERS enables rapid, accurate, and highly sensitive detection of resistant bacteria with minimal sample preparation [91]. Integrated into lab-on-a-chip (LoC) platforms, SERS enhances functionality; Lu *et al*. developed a microfluidic chip with SERS for rapid differentiation of MSSA and MRSA [102]. Chang *et al*. created a multimodal microfluidic system for on-chip bacterial enrichment, metabolite collection, and in situ SERS-based antimicrobial susceptibility testing (AST), achieving a limit of detection (LoD) of 10^3 CFU/mL [103]. Huang *et al*. 2020 reported a microfluidic platform combining SERS with microwells for low-concentration (10^3 CFU/mL) bacterial encapsulation and label-free in situ AST [104].

Despite advancements improving SERS selectivity and sensitivity, limitations persist. A drying step before analysis can cause reproducibility issues. Liquid-phase detection in natural bacterial environments is preferred but challenging due to Raman laser scattering. SERS typically analyzes single bacterial species under controlled conditions, and comprehensive SERS spectra databases for biomolecules, along with advanced mathematical processing (e.g., multivariate analysis), are still needed [105]. Ideally, SERS biosensors should enable simultaneous detection of multiple strains in complex samples.

4.3.2 Colorimetric-Based Approaches

Recent studies have developed colorimetric-based microfluidic platforms for pathogen identification and antimicrobial susceptibility testing (AST). Lee *et al*. introduced an automated microfluidic system for AST with 1-2 antibiotic combinations against bacterial pathogens, using a pH-dependent colorimetric broth to determine minimum inhibitory concentration (MIC) on-chip, with a turnaround time (TAT) of 16-24 hours. Automated fluid control (e.g., transportation, mixing) was achieved using a pneumatically controlled module, though initial sample loading (250 µL of 10^6 CFU/mL bacterial suspension per chamber) and reagents was manual [49]. Ma *et al*. developed a polymer-based microfluidic device for *Campylobacter* spp. identification and AST, featuring incubation micro-chambers with chromogenic medium and antibiotics. Bacterial growth triggered a visible colour change, enabling rapid, reliable on-chip identification and AST within 24 hours, with a limit of detection (LoD) of 10^2 CFU/mL. Variations in TAT and LoD were noted depending on the food matrix [106].

4.3.3 Quartz-Crystal Microbalance Based Approaches

Quartz-crystal microbalance (QCM) is a highly sensitive piezoelectric device capable of detecting nanogram-level changes, enabling real-time, rapid, on-site identification of antimicrobial-resistant (AMR) bacteria [107]. Reyes *et al*. 2017 developed a dynamic, accurate QCM-based system using magnesium zinc oxide (MZO) nanostructure-modified quartz crystal microbalance (MZOnano-QCM) to monitor antimicrobial effects on *Escherichia coli* and *Saccharomyces cerevisiae* and detect antimicrobial resistance genes (ARGs) [108]. Key advantages include low cost, minimal clinical sample volume, and rapid results within 10 minutes.

5. Conclusions and Future Perspectives

The global antimicrobial resistance (AMR) crisis requires close collaboration between academic researchers, pharmaceutical and diagnostics companies, government agencies, and regulatory authorities to accelerate the development of next-generation diagnostic and therapeutic solutions. A major priority is overcoming the shortcomings of existing antimicrobial susceptibility testing (AST) methods. Current technologies are hampered by lengthy and complex sample preparation requirements, insufficient sensitivity, inconsistent or incomplete pathogen identification, and limited automation, system integration, and portability. These limitations delay critical treatment decisions and contribute to the overuse of broad-spectrum antibiotics. Lengthy protocols involving culturing, isolation and identification hinder rapid pathogen detection, underscoring the need for advanced platforms with improved performance to accelerate approval and market entry. Enhancing existing methods also remains a viable strategy.

According to Markets and Markets™, the AST market is expected to reach USD 4.2 billion by 2025. Although automated AST platforms reduce incubation and detection times, their high costs limit adoption, particularly for resource-constrained institutions. Manual AST products, particularly disk diffusion, dominated the market in 2019 due to lower costs and the variety of available disks.

The discussed technologies show significant potential for tackling AMR, yet challenges persist, including their general applicability, validation against reference methods, and timelines for commercialization. Many rapid methods (minutes to hours) overlook time-consuming pre-treatment steps like culture enrichment. Standard cultivation-based AST (18-36 hours) provides minimum inhibitory concentration (MIC) but is unsuitable for non-culturable pathogens. Automated platforms (2-24 hours) may offer MIC but share this limitation. MALDI-TOF MS can determine MIC in some cases but lacks standardized protocols and companion software. Nucleic acid amplification tests (NAATs) (0.5-4 hours) are suitable for non-culturable pathogens and can integrate emerging ARGs or mutations, but they cannot determine MIC and require revalidation for updates. Whole-genome sequencing (WGS) is emerging in rapid AST but faces challenges in bioinformatics, necessitating universal databases for result interpretation. Microfluidic technologies offer versatility, minimal resource use, low cost, rapid turnaround time (TAT), automation, and portability. However, scaling fabrication for mass production and integrating user-friendly pre-treatment steps are critical for broader adoption.

No single technology fully meets rapid AST requirements, but many are poised to capture significant market share in central lab-based settings (e.g., research stations, hospitals) using WGS, whole-metagenome sequencing (WMS), PCR, MALDI-TOF MS, Fourier transform infrared spectroscopy and automated platforms, or in point-of-need (PON) settings (e.g., small labs, practitioners) with portable microfluidic devices that excel in affordability and TAT.

In conclusion, developing sensitive, reliable and affordable diagnostics is crucial in combating AMR. Rapid diagnostic tests in primary care can enable targeted treatments, while new monitoring systems, such as mobile apps and surveillance programs, are vital for tracking antimicrobial use. Emerging technologies like data mining, machine learning and automation will drive next-generation diagnostics. Epidemiological surveillance remains essential for informing therapy guidelines, antibiotic stewardship, public health interventions, and the development of new antimicrobials and vaccines. Advances in cutting-edge AMR and AST technologies, combined with enhanced surveillance for streamlined data sharing, will significantly mitigate the global AMR threat.

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Conflict of Interest

Authors has no conflict of interest.

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