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Molecular diagnostics in clinical microbiology: Advances, applications, and future directions

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ABSTRACT

Molecular diagnostics have transformed clinical microbiology by enabling rapid, accurate, and highly sensitive detection of infectious agents, significantly improving patient outcomes and public health response. Over the past decade, advances in polymerase chain reaction (PCR), next-generation sequencing (NGS), digital PCR, isothermal amplification, and CRISPR-based assays have enhanced pathogen identification, antimicrobial resistance profiling, and outbreak investigation. These technologies have been instrumental in detecting and monitoring emerging and re-emerging viral pathogens such as SARS-CoV-2, Nipah, Zika, H5N1 avian influenza, Mpox, Ebola, Marburg, and MERS-CoV. Molecular diagnostics also play a critical role in antimicrobial resistance surveillance and hospital infection control by enabling high-resolution tracking of resistance genes and pathogen transmission dynamics. Despite these achievements, challenges remain regarding implementation costs, technical expertise, infrastructure, and the need for global standardization. Future directions focus on developing cost-effective, point-of-care molecular platforms, integrating artificial intelligence and bioinformatics for enhanced interpretation, and applying these technologies within One Health and environmental surveillance frameworks. These innovations will be pivotal for early outbreak detection, real-time data-driven decision-making, and equitable access to advanced diagnostics worldwide. Ultimately, molecular diagnostics are poised to remain the cornerstone of precision medicine and infectious disease control in the era of global health challenges.

1. Introduction

Molecular diagnostics have transformed clinical microbiology, not merely by replacing traditional methods but by creating a layered diagnostic framework that aligns with escalating clinical needs. Rather than functioning as isolated tools, contemporary molecular technologies from rapid targeted NAATs to multiplex syndromic panels, CRISPR assays, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and high-resolution genomic sequencing address different stages of patient management and public

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health action [1,2].

At one end of the spectrum, rapid amplification-based assays enable timely rule-in or rule-out decisions for acute infections. Multiplex panels broaden diagnostic scope when symptoms are nonspecific, while whole-genome sequencing (WGS), metagenomics (mNGS), and One Health molecular surveillance provide deep insight for outbreak investigation, antimicrobial resistance tracking, and cross-species pathogen flow [1,2]. Collectively, these methods deliver species or strain-level information far more rapidly and accurately than conventional techniques, enhancing clinical decision-making, public health preparedness, and global infectious disease monitoring [2].

Microbial infections remain a major global health challenge, causing substantial morbidity and mortality worldwide [3]. Early and targeted antimicrobial therapy markedly improves clinical outcomes, but conventional culture-based diagnostics often require 24–48 hours or longer to yield results. These delays necessitate empirical treatment, increasing the risk of inappropriate therapy and adverse outcomes [3]. Traditional microbiological methods including culture, microscopy, and antigen detection are limited by low sensitivity, prolonged turnaround times, restricted automation, and inability to detect many fastidious bacteria and most viruses [4]. Consequently, conventional diagnostics frequently fail to provide the timely and definitive results needed for optimal patient management [4].

Molecular diagnostic technologies overcome these limitations by enabling rapid, sensitive, and specific pathogen detection directly from clinical specimens [1]. Polymerase chain reaction (PCR)-based assays amplify trace amounts of nucleic acids within hours, while modern multiplex panels can simultaneously identify multiple bacteria, viruses, or parasites [1,2]. MALDI-TOF MS provides rapid species-level identification from cultures, and next-generation sequencing (NGS) enables comprehensive, culture-independent detection of known and novel pathogens, including antimicrobial resistance determinants [2]. Real-time PCR has become the diagnostic gold standard for many viral diseases, such as HIV, influenza, and SARS-CoV-2, while nucleic acid tests have improved the detection of difficult-to-culture organisms such as *Mycobacterium tuberculosis* and *Plasmodium* species [2,4].

This review summarizes current advances in molecular diagnostic technologies including real-time and isothermal PCR, multiplex panels, MALDI-TOF MS, and NGS highlighting their applications in clinical microbiology. The roles of these technologies in detecting bacterial, viral, fungal, and parasitic pathogens, their impact on patient care, and future directions in molecular diagnostics are emphasized [1,2,4].

2. Traditional vs. molecular diagnostic approaches

2.1. Conventional culture and microscopy

Culture and microscopy remain foundational in clinical microbiology. Culture provides viable isolates for identification and antimicrobial susceptibility testing, making it the historical gold standard (Fig. 1). However, culture is slow, often requiring 24 to 72 hours or longer, and may fail to recover fastidious or non-culturable organisms [5]. For example, even rapid point-of-care urine culture requires plating specimens on agar and overnight incubation. Microscopy, including Gram stain, KOH mount, and acid-fast stain, offers immediate visual clues at low cost. Its sensitivity, however, is limited and results are operator-dependent. Microscopy can indicate the presence of bacteria or fungi but often misses low-density infections and generally cannot provide species-level identification [5].

2.2. Immunoassays

Immunoassays, which detect antibodies or antigens, bridge traditional and molecular diagnostics. Enzyme-linked immunosorbent assays (ELISAs) are widely used in centralized laboratories for detecting specific microbial antigens or host antibodies, offering high specificity and sometimes reaching femto- to attomolar sensitivity, but they require several hours, laboratory equipment, and typically assess a single analyte, limiting point-of-care use [6]. Lateral flow immunoassays (LFIAs) provide rapid results in 5 to 15 minutes, are portable and low-cost, and are applied in rapid antigen tests for influenza, malaria, and COVID-19, as well as in serological strip tests. Their main limitations are modest

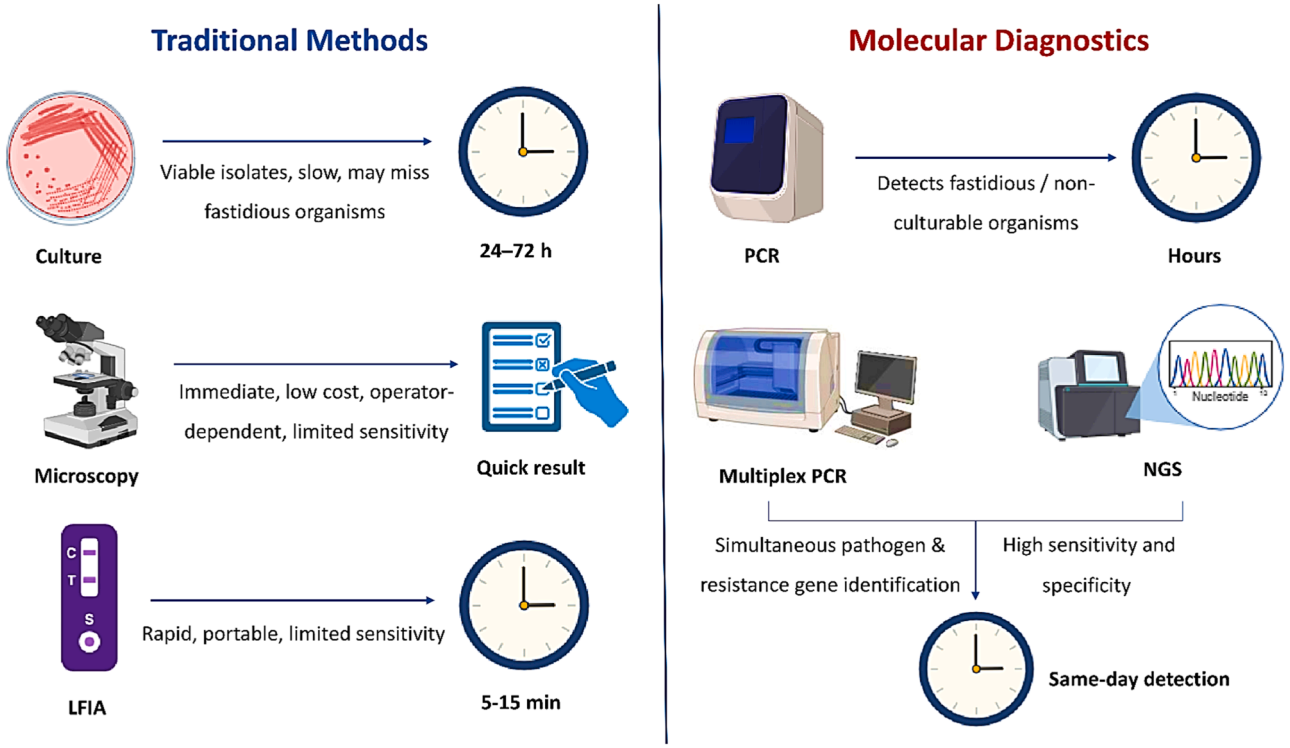

Fig. 1. Comparison of traditional and molecular diagnostic approaches in clinical microbiology.

sensitivity and qualitative readouts, detecting only high target concentrations while often missing low-abundance or low-affinity analytes; for example, COVID-19 antibody LFIAs generally achieve 70–90 % sensitivity, especially early after infection. Overall, immunoassays offer faster turnaround than culture and can be highly specific, but they are constrained by lower sensitivity (Fig. 1), potential cross-reactivity in antibody assays, and limited quantitation or multiplexing compared with molecular methods [6].

2.3. Molecular diagnostics

Molecular methods address the limitations of conventional diagnostics by directly detecting pathogen nucleic acids. PCR-based assays amplify minute amounts of DNA or RNA, allowing detection of fastidious or non-culturable organisms within hours. Multiplex PCR panels and NGS enable simultaneous identification of multiple pathogens and resistance genes, far exceeding the capabilities of culture or microscopy [7]. Clinical studies demonstrate the advantages of molecular diagnostics. In complicated urinary tract infections, PCR detected pathogens missed by culture and supported antibiotic de-escalation, improving patient outcomes. Broadly, molecular assays offer high sensitivity and specificity, rapid turnaround (often same-day), and comprehensive detection, enabling timely targeted therapy in life-threatening or difficult-to-treat infections [7]. Table 1 presents a comparative overview of diagnostic methods in clinical microbiology, highlighting their strengths and limitations.

3. PCR based diagnostics

3.1. PCR

PCR is a rapid and highly sensitive technique for detecting pathogen nucleic acids [8]. A typical amplification involves 30 to 40 thermal cycles, producing detectable products within a few hours. Conventional or endpoint PCR employs pathogen-specific primers, such as bacterial 16S rRNA or parasitic 18S rRNA genes, to amplify DNA, with products visualized by gel electrophoresis. Real-time PCR (qPCR) improves upon this approach by enabling simultaneous amplification and detection through fluorescence-based probes or dyes (Fig. 2). It provides quantitative information by measuring fluorescence intensity during each cycle, completing within about one to two hours. qPCR eliminates post-amplification handling, reduces contamination risk, and quantifies pathogen load via cycle threshold values, allowing both detection and monitoring of infection dynamics. Conventional PCR generally detects around 10^2 to 10^3 copies of target DNA, whereas qPCR achieves single-digit detection limits, typically 1 to 10 copies per reaction, with a wide dynamic range. Multiplex RT-qPCR assays for respiratory viruses such as SARS-CoV-2 and influenza demonstrate sensitivities of 95 % or higher, with detection limits near 4 to 5 copies per reaction [9]. PCR can target conserved or species-specific genes, including **16S rRNA, *rpoB*,** and ***gyrA*** for bacteria; **N, E**, or **RdRP** for coronaviruses; **M** for influenza; fungal **rRNA** or **ITS** regions such as *Aspergillus* 18S; and parasitic **18S rRNA** genes, such as those of *Plasmodium* species [10]. Compared with culture and microscopy, PCR and qPCR provide faster results and higher sensitivity. For instance, qPCR assays for fungal pathogens achieve sensitivities up to 90 % relative to culture, while multiplex qPCR panels can identify bloodstream pathogens within hours, supporting rapid, targeted therapy. However, PCR detects nucleic acids regardless of organism viability, so results must be interpreted in conjunction with clinical findings to distinguish active infection from residual or colonizing DNA [9].

Table 1
Comparative overview of diagnostic methods in clinical microbiology: strengths and limitations.

Method	Strengths	Limitations
Culture	Gold-standard identification, antibiotic susceptibility testing	Slow (days to weeks), may miss fastidious organisms
Microscopy	Immediate, low-cost screening	Operator-dependent, low sensitivity, limited species identification
ELISA	High specificity and sensitivity, quantitative	Requires hours, lab instrumentation, not point-of-care
Lateral Flow	Rapid, portable, inexpensive, useful for screening	Qualitative, limited sensitivity for low-abundance targets
Molecular Tests	Very high sensitivity and specificity, rapid, detects non-culturable pathogens and resistance genes	Requires specialized equipment, higher cost

3.2. Multiplex PCR for pathogen panels

Multiplex PCR assays employ multiple primer and probe sets within a single reaction to detect several pathogens simultaneously [11]. Each target is labeled with a distinct fluorescent probe, such as TaqMan or molecular beacons, allowing parallel detection and differentiation of multiple organisms in one test [11]. Commercial syndromic panels, including the **BioFire FilmArray** and **Seegene Allplex systems**, exemplify this approach by targeting 10 to more than 20 pathogens per assay [12]. For instance, the FilmArray Respiratory Panel 2.1 detects 23 respiratory pathogens, including 19 viruses such as SARS-CoV-2, along with *Bordetella* and *Chlamydia* species, while the Allplex GI-Parasite assay identifies *Giardia, Entamoeba*, and *Cryptosporidium* species in stool with approximately 100 % sensitivity and specificity.

Multiplex PCR significantly reduces diagnostic turnaround time while expanding pathogen coverage. Instead of performing serial single-target tests, a single multiplex run typically completed within one to two hours, can yield comprehensive results. This capability is particularly valuable in acute infections such as sepsis, meningitis, or pneumonia, where early identification supports prompt, targeted therapy [10]. Reported sensitivities of multiplex quantitative PCR (qPCR) assays are comparable to those of singleplex PCR. For example, a quadruplex RT-qPCR assay detecting SARS-CoV and influenza A and B achieved limits of detection of approximately 4–5 copies per reaction in 35 minutes, comparable to reference assays. Clinically, multiplex PCR panels have substantially improved the speed and precision of infectious disease diagnosis. In bloodstream infections, multiplex PCR can reduce time to identification from 2–3 days with culture to 4–6 hours, enabling earlier initiation of targeted antimicrobial therapy and supporting antibiotic stewardship through simultaneous detection of resistance genes. In fungal diseases, panels targeting *Candida* and *Aspergillus* species provide faster and more sensitive detection than culture, and multiplex gastrointestinal assays show near-perfect concordance with microscopy for protozoan pathogens. Despite these advantages, multiplex assays require meticulous optimization to prevent primer-dimer formation and cross-reactivity. They are costlier than singleplex tests and require specialized equipment and trained personnel, which can limit adoption in low-resource laboratories. Furthermore, contamination can result in false positives, underscoring the importance of stringent assay design and workflow controls [10]. Table 2 presents a comparison of commercially available multiplex PCR pathogen panels, highlighting their target range, turnaround time, analytical performance, and clinical applications.

In addition to commercially available molecular assays, many large hospitals, academic centers, and reference laboratories rely extensively on laboratory-developed tests (LDTs) to meet diagnostic needs that are not fully addressed by commercial kits. LDTs offer substantial flexibility in assay design, enabling rapid adaptation to emerging pathogens, newly discovered resistance genes, and locally relevant epidemiological patterns [13]. They may also reduce costs by allowing laboratories to optimize workflows, reagents, and batch sizes according to clinical demand. However, the implementation of LDTs requires rigorous

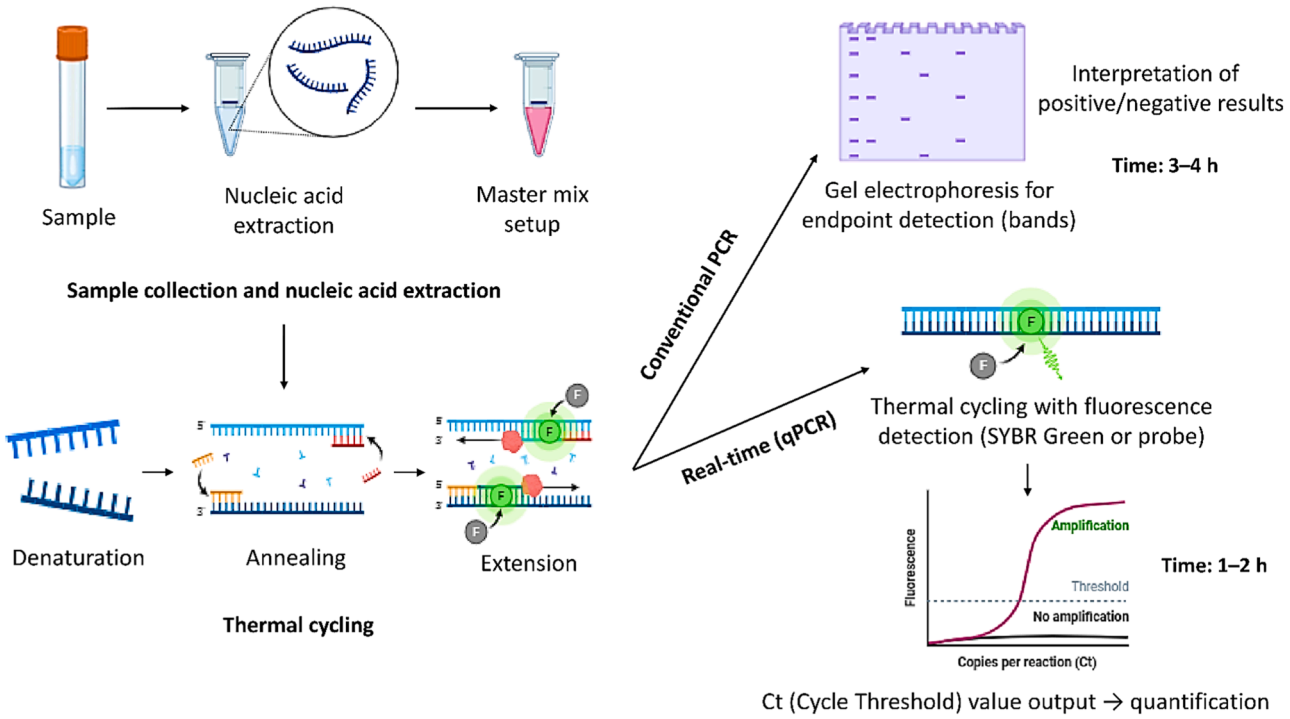

Fig. 2. Overview of Thermal Cycling and detection differences between conventional PCR and Real-Time PCR (qPCR).

Table 2
Comparison of commercial multiplex PCR pathogen panels.

System/Manufacturer	Panel Type	Number of Targets	Representative Pathogens	Turnaround Time	Sensitivity/ Specificity	Clinical Application
BioFire FilmArray RP 2.1 (bioMérieux)	Respiratory	23 (19 viruses + 4 bacteria)	SARS-CoV-2, Influenza A/B, RSV, *Bordetella spp.*	~1 hr	≥95–100 %	Respiratory infections
Seegene Allplex GI-Parasite	Gastrointestinal	3 protozoa	*Giardia lamblia, Entamoeba histolytica, Cryptosporidium spp.*	~2 hr	~100 %	GI parasitic infections
QIAstat-Dx Panel (Qiagen)	Respiratory / GI	20–25	Mixed viral/bacterial	1.5 hr	≥95 %	Syndromic testing
Luminex NxTAG Respiratory Pathogen Panel	Respiratory	18–20	*Influenza A/B, Parainfluenza, Adenovirus*	2 hr	≥95 %	Respiratory diagnostics

validation, ongoing quality control, and well-defined performance monitoring to ensure clinical reliability. Regulatory oversight frameworks differ across countries and continue to evolve, posing additional challenges regarding standardization and compliance. Despite these constraints, LDTs remain a critical component of the molecular diagnostics ecosystem, complementing commercial platforms and supporting timely, context-specific infectious disease testing [14].

3.3. Pathogen specific PCR applications

3.3.1. Bacterial infections

PCR and qPCR have revolutionized bacterial diagnostics by enabling rapid, highly sensitive detection of pathogens directly from clinical specimens [15]. Broad-range **16S rRNA PCR** can identify virtually any bacterial species present in sterile-site samples, making it invaluable in cases such as culture-negative endocarditis and osteomyelitis. Species-specific qPCR assays, such as *M. tuberculosis* IS6110 detection, provide results within hours, a major improvement over the weeks required for traditional culture (Fig. 3). Multiplex blood-culture PCR panels, approved by the FDA, can simultaneously detect key sepsis-related pathogens, including *Staphylococcus aureus, Escherichia coli*, and *Enterococcus* species. In practice, qPCR can identify as few as 10^1–10^2 colony-forming units (CFU) per millilitre of blood, whereas standard culture typically requires bacterial loads exceeding 10^4 CFU/mL for detection [15].

Advances in multiplex droplet digital PCR (ddPCR) have further improved the analytical sensitivity and quantitative accuracy of pathogen detection. In a recent clinical study involving patients with suspected sepsis, an 18-plex ddPCR assay detected pathogens in 53 % of specimens compared with 28 % detected by conventional culture; however, these figures represent overall detection rates rather than validated diagnostic accuracy measures [15]. The reported ~89 % sensitivity was calculated only among culture-positive samples, and corresponding specificity values were not provided in the original study [15]. Importantly, ddPCR identified pathogens in an additional 25 % of culture-negative samples. Although this may reflect the superior analytical sensitivity of ddPCR supported by measured DNA concentrations ranging from 30 to 3 × 10^5 copies/mL these additional detections cannot be assumed to indicate true infection [15]. Such findings may result from early-phase infection prior to culture positivity, detection of non-viable organisms or residual nucleic acids, colonization, or potential false-positive results. Consequently, molecular "positives" in culture-negative samples require careful clinical interpretation, integrating symptomatology, inflammatory markers, imaging findings, and therapeutic response to determine their clinical relevance. This strengthened analysis presents ddPCR performance with appropriate caution, underscoring both its potential diagnostic value and its interpretive limitations, while acknowledging that conventional PCR continues to provide rapid and clinically actionable results for many bacterial pathogens [15].

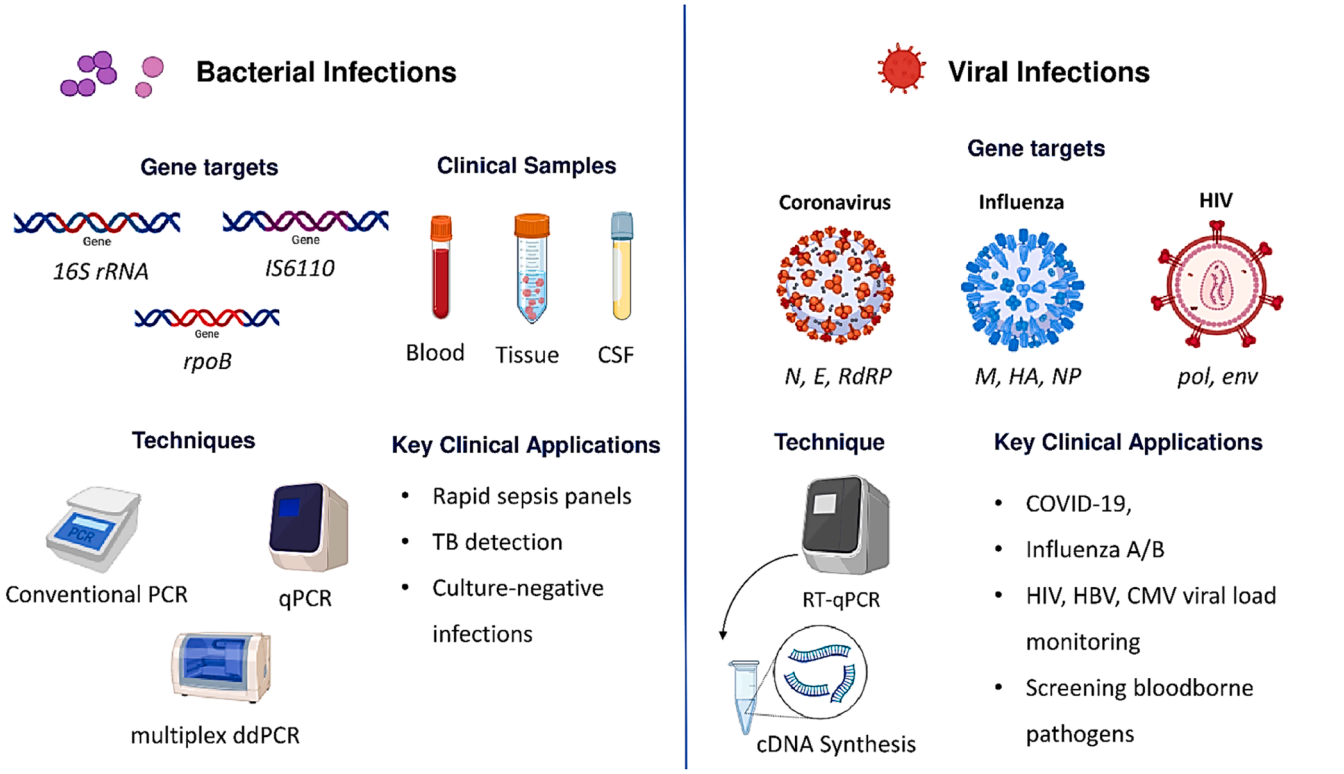

Fig. 3. PCR based detection strategies for bacterial and viral pathogens.

3.3.2. *Viral infections*

Reverse transcription PCR (RT-PCR) remains the gold standard for detecting viral pathogens, particularly RNA viruses such as SARS-CoV-2, influenza, HIV, and HCV [10,16,17]. In this method, viral RNA is first converted into complementary DNA (cDNA) through a reverse transcription step, followed by quantitative PCR (qPCR) amplification. Assays typically target conserved genomic regions such as the **N, E,** or **RdRP** genes for coronaviruses; **M, HA**, or **NP** for influenza; and **pol** or **env** for HIV, to ensure high specificity and broad strain coverage (Fig. 3). Well-optimized RT-qPCR assays can achieve near single-copy detection limits. For instance, an ultrafast quadruplex RT-qPCR assay was able to differentiate SARS-CoV-2 and influenza A/B within 35 minutes, achieving limits of detection around 4–5 copies per reaction [10].

Standard RT-qPCR protocols generally run 40–50 amplification cycles and produce results within one to two hours using high-speed polymerases. The sensitivity of RT-qPCR far surpasses that of antigen tests or culture methods; while antigen assays typically require 10^3–10^5 RNA copies per microliter, RT-qPCR can reliably detect fewer than one copy per microliter. Clinically, RT-qPCR serves multiple roles, acute viral diagnosis (e.g., COVID-19 testing), viral load monitoring (HIV, HBV, CMV), and screening for bloodborne pathogens. Multiplex RT-qPCR panels have further expanded diagnostic capacity by detecting multiple viruses in a single reaction. For example, comprehensive respiratory panels can simultaneously identify up to 23 pathogens, including SARS-CoV-2, thereby facilitating rapid and accurate diagnosis of co-infections and improving clinical decision-making [11].

3.3.3. *Fungal infections*

Molecular diagnostics have substantially advanced the detection of invasive fungal infections, which are often difficult to identify using conventional culture methods [9]. PCR-based assays typically target conserved fungal genomic regions such as **18S** and **28S rRNA genes** or **internal transcribed spacer (ITS)** regions, as well as genus or species-specific genes. Quantitative PCR (qPCR) assays are now widely applied for major fungal pathogens, including *Aspergillus, Candida, Pneumocystis jirovecii*, Mucorales, and dimorphic fungi. Multiple studies and meta-analyses have demonstrated that fungal qPCR achieves high diagnostic sensitivity, frequently exceeding 80–90 % and specificity that surpasses culture and microscopy. For instance, *Aspergillus* PCR in bronchoalveolar lavage fluid shows reported sensitivities of 57–91 % and specificities of 92–97 % [9].

Beyond improved accuracy, PCR provides rapid results within hours, enabling earlier diagnosis and treatment. In cases of candidemia, qPCR can detect *Candida* DNA approximately two hours before blood cultures become positive, which often requires several days [9]. Consequently, fungal PCR is increasingly incorporated into diagnostic workflows alongside antigen detection assays such as galactomannan and β-D-glucan. A negative fungal PCR, given its high sensitivity, can serve as a strong indicator to exclude invasive infection in low-risk patients. However, limitations remain: false positives may arise from environmental contamination or colonization, emphasizing the importance of quantitative interpretation using cycle-threshold (Ct) values and confirmatory or repeat testing to ensure diagnostic accuracy [9].

3.3.4. *Parasitic infections*

PCR has also markedly improved the diagnosis of parasitic infections, offering greater sensitivity and faster turnaround than conventional microscopy or antigen detection [18]. In malaria diagnostics, real-time PCR assays commonly target the multicopy **18S rRNA gene** (5–7 copies per *Plasmodium* genome) to enable precise species identification. Multiplex PCR platforms can simultaneously detect all five human *Plasmodium* species in a single reaction. These assays demonstrate extremely low detection limits, for example, validated multiplex qPCR can detect *P. vivax* at 0.25 parasites/μL, *P. falciparum* and *P. knowlesi* at 0.5 parasites/μL, and *P. malariae* at 5 parasites/μL values far below the ~50–100 parasites/μL threshold of microscopy. In external quality assessments, such assays have achieved 100 % sensitivity and specificity. Results are typically available within 1–2 hours, making PCR particularly valuable for detecting low-parasitemia or mixed-species infections that are easily missed by routine tests [18].

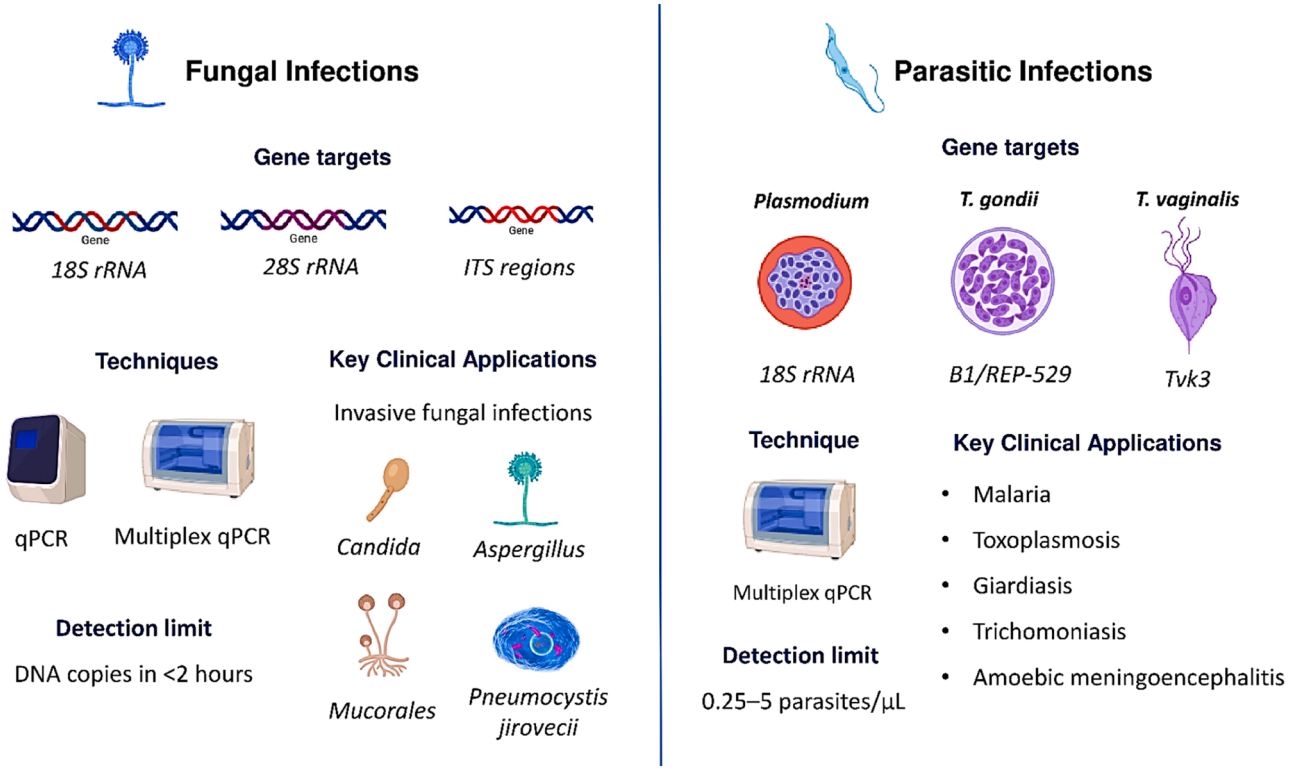

Fig. 4. Molecular diagnostic applications of PCR and qPCR in fungal and parasitic Infections.

(Fig. 4).

For intestinal protozoa, multiplex qPCR panels such as **Allplex™ GI-Parasite Assay** enable concurrent detection of *Giardia, Entamoeba, Dientamoeba*, and *Cryptosporidium* species [12]. A multicenter study in Italy demonstrated nearly 100 % sensitivity and specificity for *Giardia, Cryptosporidium*, and *Entamoeba histolytica*, with clear superiority over microscopy. Other PCR assays target *Toxoplasma gondii* (**B1 or REP-529 genes in amniotic fluid**) or *Trichomonas vaginalis* (**Tvk3 sequence**), providing accurate, rapid confirmation of infection [12]. Overall, parasite PCR enables detection down to a few gene copies or fractions of a parasite, facilitating early and subclinical diagnosis. However, its implementation remains constrained by cost, reagent requirements, and the lack of FDA-approved assays for many helminthic infections, where microscopy and serology continue to play complementary roles [12].

4. NGS in Microbiology

4.1. Whole-genome sequencing (WGS)

WGS has transformed microbial genomics, epidemiology, and

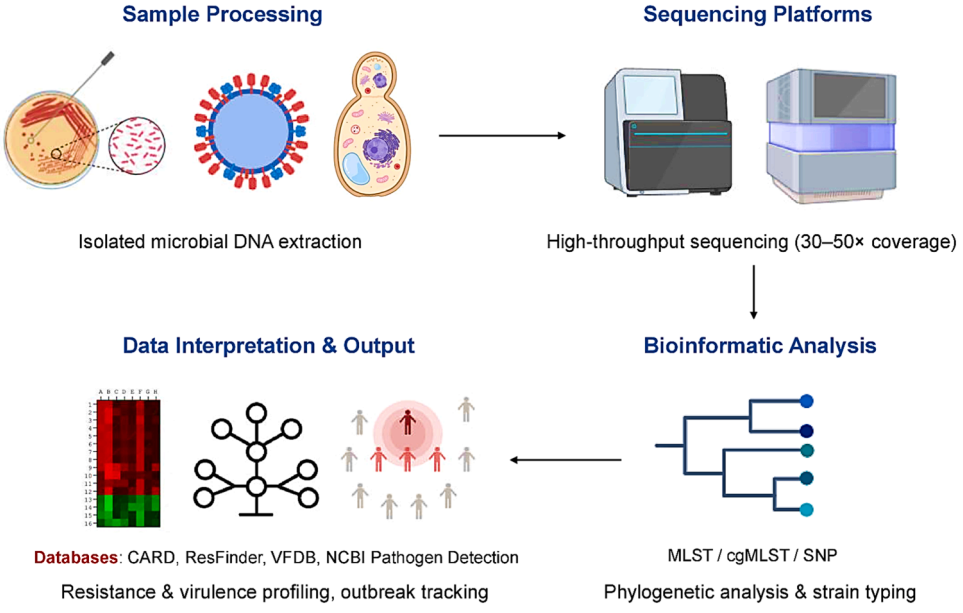

Fig. 5. Overview of whole-genome sequencing (WGS) in clinical microbiology.

antimicrobial resistance surveillance by enabling complete characterization of pathogen genomes at single-nucleotide resolution [19]. In WGS, genomic DNA from a bacterial, viral, or fungal isolate is fragmented and sequenced, commonly using Illumina short-read or long-read platforms such as **Oxford Nanopore** or **PacBio**. The reads are then assembled or aligned against reference genomes to reconstruct the organism's full sequence. Deep sequencing coverage (typically ≥30–50× for bacteria) ensures accurate variant detection and reliable gene identification. Resulting genome assemblies are compared with public or internal databases such as PulseNet, TESSy, or NCBI Pathogen Detection to assign multilocus sequence types (MLST, cgMLST) or identify single-nucleotide polymorphism (SNP) profiles for phylogenetic analysis (Fig. 5). These genomic signatures enable precise tracking of strain relationships across patients, institutions, and geographic regions. In outbreak investigations, cgMLST and SNP-based analyses can discriminate closely related isolates, revealing transmission links that conventional typing methods (e.g., PFGE or PCR) often fail to detect. Standardized pipelines now facilitate interlaboratory data sharing and real-time outbreak surveillance, greatly enhancing global microbial monitoring [19].

Beyond strain typing, WGS allows comprehensive profiling of antimicrobial resistance (AMR) and virulence factors [19,20]. Sequence data are screened against curated resistance and virulence gene databases such as CARD, ResFinder, and VFDB to identify genetic determinants and predict phenotypic resistance. This genome-wide approach provides a detailed picture of the resistome and mobilome within a single sequencing run [19]. Multiple studies show that concordance between WGS-based antimicrobial resistance (AMR) prediction and phenotypic susceptibility testing varies widely across organism–drug pairs, rather than achieving uniformly high accuracy. High performance is generally seen for well-characterized resistance determinants such as carbapenemase genes in *Acinetobacter baumannii* and Enterobacterales, or mecA-mediated methicillin resistance in *S. aureus* where genotype reliably predicts phenotype. In contrast, accuracy is substantially lower for drug classes with complex or incompletely defined resistance mechanisms, including colistin, some aminoglycosides, and agents affected by regulatory mutations or novel gene variants absent from current databases. These gaps highlight that WGS alone cannot reliably detect emerging or expression-dependent resistance mechanisms without phenotypic support. Even so, WGS remains central to clinical and public health practice, providing high-resolution genomic data for pathogen identification, transmission mapping, and tracking the spread of AMR determinants, and serving as a cornerstone of modern molecular surveillance programs [20].

4.2. *Metagenomics*

Metagenomic next-generation sequencing (mNGS), also known as **shotgun metagenomics**, enables unbiased pathogen detection directly from clinical specimens without the need for culture or prior target selection [21]. In this approach, all nucleic acids extracted from a sample such as blood, cerebrospinal fluid, or respiratory secretions are randomly sequenced (Fig. 6), generating millions of short reads derived from both host and microbial genomes. Bioinformatic algorithms subsequently classify these reads, identifying microbial sequences amid the abundant human background. Unlike targeted PCR assays, mNGS is hypothesis-free and can detect any bacterial, viral, fungal, or parasitic DNA or RNA present above the analytical threshold. This broad scope makes mNGS particularly valuable for diagnosing infections of unknown etiology, such as **meningitis, encephalitis, pneumonia**, or **fever of unknown origin (FUO)**, where conventional testing fails to identify a causative agent. For instance, long-term clinical studies of cerebrospinal fluid samples have shown that mNGS can detect pathogens in a significant proportion of previously undiagnosed encephalitis cases [21,22].

Beyond detecting known organisms, mNGS can identify novel or highly divergent pathogens. Advanced bioinformatic workflows employing *de novo* assembly and protein-level homology searches enable recognition of agents with minimal sequence similarity to existing database entries [23]. In one FDA-authorized study, an mNGS assay detected more than 90 % of known respiratory viruses in clinical samples with 93.6 % sensitivity and successfully identified hundreds of divergent viral strains *in silico* without prior primer information. Even when human reads were excluded from reference databases, the assay correctly classified all 70 test human viruses based on protein homology to related animal viruses, demonstrating its capacity to flag unexpected or emerging pathogens [22,23].

Turnaround time for mNGS depends on workflow complexity. Optimized protocols using nanopore or streamlined Illumina sequencing

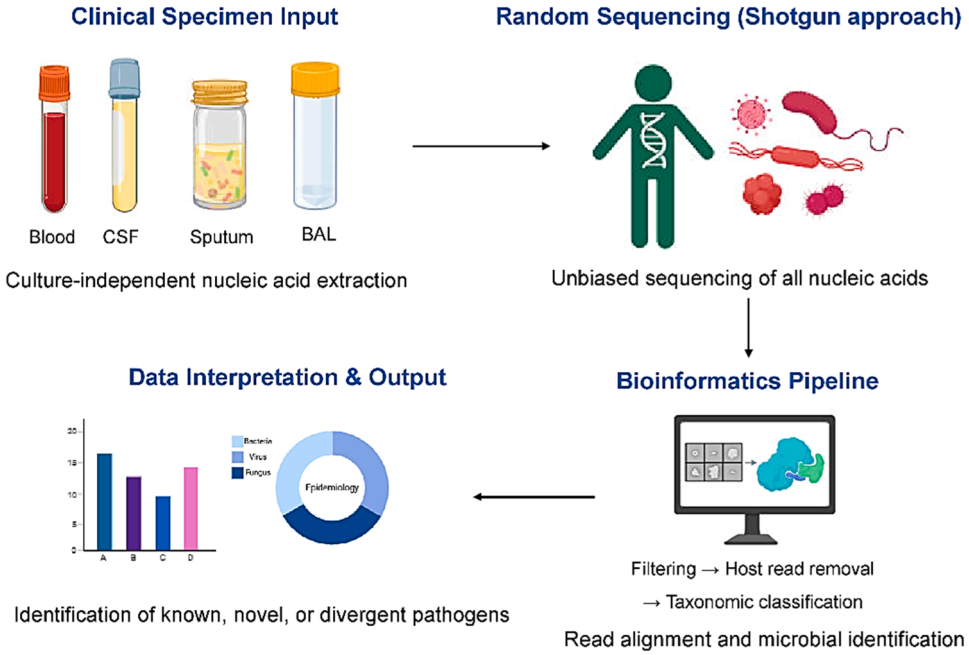

Fig. 6. Metagenomic NGS (mNGS) for unbiased pathogen detection.

can deliver results within 24 hours, suitable for acute respiratory or sepsis diagnostics [23]. However, in typical clinical settings, including centralized laboratory workflows, total turnaround often extends to several days. For example, a major central nervous system mNGS program reported a median of 3.6 days from sample receipt to result, extending to 8–11 days when transport and reporting were included. Despite these logistical constraints, mNGS provides unmatched diagnostic breadth and resolution, establishing it as a key technology for comprehensive pathogen discovery, outbreak investigation, and emerging infectious disease surveillance [22,23]. Table 3 depicts a simplified comparison between WGS and mNGS, highlighting their key differences in sample type, approach, applications, and limitations.

4.3. Clinical applications

WGS has become the gold standard for microbial epidemiology and outbreak investigation. By comparing entire genomes, WGS can delineate transmission chains and identify outbreak sources with far greater precision than conventional typing methods [24]. Large-scale implementation by public health agencies including the **CDC, ECDC**, and **PulseNet** has made WGS routine for surveillance of *Salmonella, Listeria monocytogenes, E. coli, S. aureus* (MRSA) (Fig. 7), and other clinically relevant pathogens [19]. Sequencing-based surveillance enables rapid detection of related isolates and uncovering of hidden transmission events that might otherwise go unnoticed. Once sequencing infrastructure is established, outbreak clusters can be confirmed within hours to days. Typical sequencing depths of 30–50× are sufficient for accurate phylogenetic analysis, although higher coverage is often used in critical outbreak investigations to ensure robust variant calling [24,25].

Beyond outbreak tracing, WGS has substantially advanced AMR surveillance (Fig. 7). By identifying resistance genes and mutations across entire genomes, **WGS-based antimicrobial susceptibility testing (WGS-AST)** can predict resistance phenotypes (Fig. 7) before culture results are available. Portable sequencing platforms such as **Oxford Nanopore** now permit same-day detection of carbapenemase and extended-spectrum β-lactamase (ESBL) genes directly from isolates or clinical samples. Consequently, many reference and clinical laboratories are transitioning toward WGS-integrated surveillance systems that provide comprehensive resistome profiles and improve antibiotic stewardship [24].

NGS technologies also facilitate pathogen discovery and diagnosis in unexplained infections (Fig. 7). mNGS has identified unexpected or novel pathogens in complex clinical cases, such as *West Nile virus* in encephalitis or *Leptospira* in culture-negative meningitis. Its unbiased design allows detection of any microbial nucleic acid in a specimen, making it invaluable for investigating outbreaks of unknown etiology or atypical infections in immunocompromised hosts. During the **COVID-19 pandemic, NGS played a critical role in identifying SARS-CoV-2 and monitoring emerging variants globally** (Fig. 7). Comparative studies show that mNGS can reveal pathogens missed by conventional diagnostics, contributing additional findings in 14–35 % of undiagnosed cases depending on specimen type and clinical setting [26].

Molecular assays are now central to diagnosing bacterial, viral, fungal, and parasitic infections, shortening time to targeted therapy and supporting antimicrobial stewardship [2,4]. High-quality evidence demonstrates clinical benefits: in bloodstream infections, rapid multiplex PCR from positive blood cultures with stewardship support reduces time to effective therapy, accelerates de-escalation of broad-spectrum antibiotics, and limits unnecessary treatment of contaminants [27,28]. A meta-analysis of 88 studies (>25,000 patients) reported reduced mortality (OR 0.72, 95 % CI 0.59–0.87), shorter hospital stays, and ~29-hour faster time to optimal therapy [29]. For respiratory viral infections, syndromic PCR panels improve initial antimicrobial adequacy and reduce time to effective treatment by ~28 hours, though effects on antibiotic prescribing and hospitalization vary [30]. While robust evidence exists for infections such as influenza and severe pneumonia, data remain limited for some populations and newer molecular platforms, underscoring the need for further high-quality outcome studies.

Table 3
Comparison between whole-genome sequencing (WGS) and metagenomic next-generation sequencing (mNGS).

Feature	WGS (Whole-Genome Sequencing)	mNGS (Metagenomic Sequencing)
Sample	Needs a pure cultured isolate	Uses direct clinical samples (blood, CSF, etc.)
Target	One organism's full genome	All DNA/RNA in the sample
Method	Targeted sequencing	Unbiased or shotgun sequencing
Use	Typing, AMR and virulence study, outbreak tracing	Detects known and unknown pathogens
Output	Complete genome of one organism	Mixed sequences from many organisms
Data analysis	Compare with reference genomes	Classify reads and find microbes
Applications	Epidemiology and resistance surveillance	Diagnosis of unknown infections
Time	Usually 1–2 days	Usually 1–5 days
Need for culture	Yes	No
Main limitation	Works only if organism is cultured	Costly and data analysis is complex

5. CRISPR-based diagnostics

CRISPR-based diagnostics such as **SHERLOCK (Cas13a)** and **DETECTR (Cas12a)** use RNA-guided nucleases to identify pathogen nucleic acids and cleave reporter molecules to generate a detectable signal. These assays typically pair CRISPR detection with isothermal amplification (RPA or LAMP) to produce sufficient target DNA or RNA. Upon target recognition, Cas12 or Cas13 activates collateral cleavage of a labeled reporter, enabling attomolar sensitivity and single-nucleotide specificity for viral and bacterial pathogen detection (Fig. 8). Emerging enzymes such as **Cas14** which offers exceptional specificity for single-stranded DNA targets and Cas3 whose helicase-nuclease activity provides robust signal amplification through processive DNA degradation are also being explored to expand the diagnostic capabilities and flexibility of CRISPR platforms. DETECTR can detect SARS-CoV-2 RNA within approximately 30 minutes with a detection limit near 10 copies/μL, comparable to RT-PCR, while SHERLOCK achieves attomolar sensitivity for Dengue and Zika viruses and can operate without nucleic acid extraction, making it suitable for low-resource settings [31].

Recent advances have moved CRISPR diagnostics from experimental tools to clinically validated platforms. In 2020, the FDA granted Emergency Use Authorization (EUA) to the Sherlock Biosciences CRISPR based SARS-CoV-2 assay, the first CRISPR diagnostic to receive regulatory authorization, demonstrating the feasibility of deploying CRISPR technology in clinical settings [32]. The Sherlock CRISPR SARS-CoV-2 test showed analytical sensitivity down to 6.75 copies/μL with ≥97 % concordance compared with RT PCR, while DETECTR based SARS-CoV-2 assays demonstrated limits of detection around 10 copies/μL and 93–98 % agreement with quantitative RT PCR in peer reviewed evaluations. Beyond COVID-19, clinical pilot studies applying CRISPR platforms to tuberculosis, HPV genotyping, and antimicrobial resistance markers report performance comparable to PCR, with detection of low copy targets in under an hour. Collectively, these findings highlight that CRISPR diagnostics, when integrated with isothermal amplification, can achieve sensitivity and specificity approaching those of gold standard PCR, while offering faster turnaround, minimal instrumentation, and strong potential for decentralized clinical testing [32–34].

CRISPR assays combine high sensitivity, rapid turnaround, and portability. Many yield results in under an hour due to the integration of

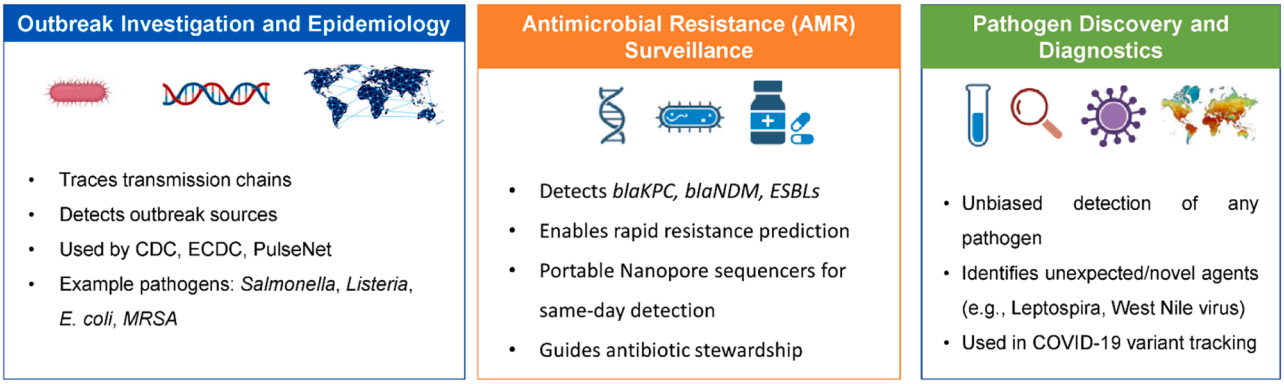

Fig. 7. Clinical applications of WGS and mNGS in clinical microbiology.

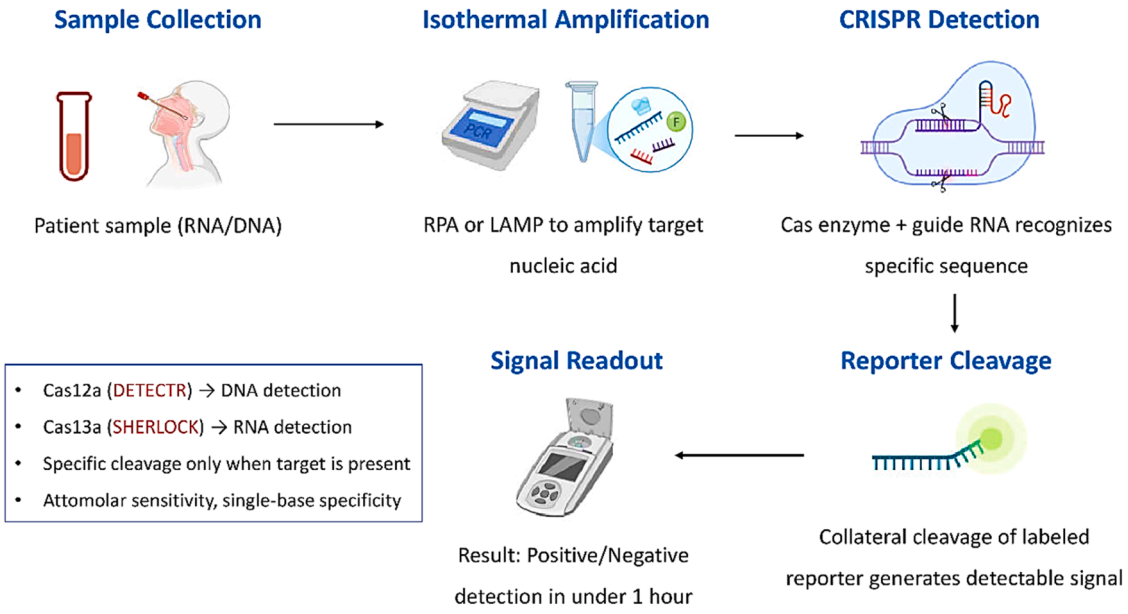

Fig. 8. Workflow of CRISPR-based nucleic acid detection assay.

isothermal amplification and immediate Cas-mediated signal generation. They require only simple equipment, such as a constant-temperature heater and a fluorescence or lateral-flow readout, and can be lyophilized for field use. This minimal instrumentation and robustness make CRISPR diagnostics promising tools for rapid, point-of-care testing in both clinical and outbreak settings [31].

6. Isothermal amplification techniques

Isothermal methods obviate the need for thermocyclers by amplifying nucleic acids at a constant temperature, enabling fast, field-friendly assays.

6.1 LAMP (loop-mediated isothermal amplification)

LAMP uses 4–6 primers targeting 6–8 regions of the DNA and a strand-displacing polymerase (e.g., **Bst**) at ~60–65 °C. It rapidly generates large "dumbbell" or "loop" DNA concatemers, often >20 kb, producing visible byproducts such as magnesium pyrophosphate turbidity or strong fluorescence. Highly robust, LAMP tolerates inhibitors, allowing amplification from crude samples with minimal purification. Detection can be simple, colorimetric dyes or lateral-flow strips often allow results to be read by eye. Requiring only a heating block or water bath, LAMP is widely used in portable diagnostics, with assays developed for pathogens including SARS-CoV-2, Zika, and filariasis [35].

6.2 RPA (recombinase polymerase amplification)

RPA amplifies DNA (or RNA via RT-RPA) at low temperatures (~37–42 °C) using a **recombinase, single-stranded binding proteins**, and a **strand-displacing polymerase**. Primers invade double-stranded DNA to initiate amplification, often achieving 10^6 fold amplification in under 30 minutes. Its near-body-temperature operation allows use with simple heaters or portable devices. Highly sensitive and fast, RPA can detect target nucleic acids in 10–20 minutes. Like LAMP, RPA has been incorporated into rapid tests and lateral-flow assays, and its tolerance of crude samples makes it ideal for field diagnostics of neglected or emerging pathogens [36]. Table 4 depicts a comparison between LAMP and RPA.

7. Digital PCR and high-throughput platforms

Digital PCR (dPCR) partitions samples into thousands of nanoliter-scale reactions (e.g., droplets or chip wells), allowing individual target molecules to be amplified independently [37]. This architecture enables absolute nucleic acid quantification without reliance on standard curves, providing substantially higher precision than qPCR and making

Table 4
Comparison of LAMP and RPA isothermal amplification techniques.

Feature	LAMP (Loop-Mediated Isothermal Amplification)	RPA (Recombinase Polymerase Amplification)
Reaction Temperature	60–65°C	37–42°C
Key Enzymes	Bst DNA polymerase	Recombinase, SSBs, strand-displacing polymerase
Primers	4–6 primers targeting 6–8 regions	2 primers (optional probes for fluorescence)
Amplification Time	30–60 min	10–30 min
Detection	Colorimetric dyes, turbidity, fluorescence, or lateral flow	Fluorescence or lateral-flow assays
Sample Tolerance	Tolerant to inhibitors; crude samples usable	Tolerant to inhibitors; minimal purification
Equipment Needed	Simple heater or water bath	Simple heater or portable incubator
Applications	Pathogen detection (SARS-CoV-2, Zika, filariasis)	Rapid field diagnostics, emerging pathogens
Advantages	Robust, visible readouts	Fast, low-temperature operation

dPCR particularly valuable for monitoring viral load dynamics in infections such as HIV and HBV, where accurate quantification is essential for treatment decisions. Its precision also underpins clinical applications such as post-transplant chimerism assessment, where small shifts in donor–recipient DNA fractions must be reliably detected. dPCR is highly sensitive to low-abundance targets, capable of detecting rare variants at ≈0.1–1 % frequency that conventional PCR may miss. For example, droplet dPCR detected *E. coli* DNA in 82.7 % of confirmed bloodstream infections (versus 0 % in negatives), demonstrating superior sensitivity over qPCR. In AMR surveillance, dPCR quantifies resistance gene copy numbers in clinical and environmental samples, with reported levels of 10^3–10^4 copies/ng for sulfonamide and tetracycline genes in sewage. Overall, dPCR offers precise microbial load measurement and robust detection of low-level infections or rare resistant subpopulations, strengthening its role in both clinical diagnostics and surveillance systems [38].

Modern diagnostics also use multiplexed, high-throughput approaches to analyze many targets or samples simultaneously. Microfluidic qPCR systems (e.g., **Biomark HD**) can run dozens of TaqMan assays in parallel; a recent wastewater study used a 48.48 dynamic array to quantify 36 targets including viral (norovirus GII), bacterial (*Salmonella, Campylobacter*), and ARG markers (*sul1, int1, bla CTX-M*) across multiple samples, finding 100 % positivity for some ARGs. Similarly, NGS and high-density microarrays profile entire microbial communities and resistomes in a single run. Combined with dPCR quantification, these high-throughput methods enable large-scale pathogen and resistance gene monitoring for public health and surveillance programs [39]. Table 5 summarizes molecular diagnostic technologies, highlighting their optimal clinical applications, turnaround times, strengths, and limitations.

8. Applications in clinical and public health settings

8.1. Emerging viral pathogens

8.1.1. Nipah virus (NiV)

NiV causes severe zoonotic outbreaks with high mortality, so rapid molecular detection is critical. Real-time reverse-transcription PCR (qRT-PCR) assays targeting the viral N gene are the gold standard for acute infection, achieving detection limits of ~10^3–10^4 RNA copies per reaction. Clinical specimens such as nasal swabs, CSF, urine, and blood are used for testing. Early diagnosis by qRT-PCR is essential for outbreak control and patient care, and there is ongoing work to develop rapid point-of-care NiV molecular tests to improve surveillance in endemic regions [40].

8.1.2. Zika virus

During recent Zika outbreaks (Fig. 9), molecular assays were essential for case confirmation. Real-time RT-PCR of viral RNA in serum or urine remains the primary diagnostic method. Multiplex PCR panels, such as the **CDC Trioplex assay**, detect Zika, dengue, and chikungunya viruses simultaneously with high sensitivity and specificity, even in outbreak settings [41]. In one field study, the Trioplex RT-PCR reliably detected Zika RNA alongside dengue during an outbreak, underscoring its value as a clinical screening tool [41]. Novel isothermal and CRISPR-based NAATs are also being developed to enable point-of-care testing in resource-limited settings, though these extend beyond the classical PCR focus here [41].

8.1.3. SARS-CoV-2 (COVID-19)

The COVID-19 pandemic drove unprecedented use of molecular diagnostics. RT-qPCR targeting viral genes such as **N, E**, and **ORF1ab** remains the reference standard for detecting SARS-CoV-2 RNA in respiratory specimens [42]. Although it requires specialized laboratories, RT-qPCR's high sensitivity has made it indispensable for clinical diagnosis and public health surveillance [42]. In parallel, high-throughput genome sequencing has been deployed globally to monitor emerging variants. Real-time sequencing enabled identification of novel SARS-CoV-2 lineages, including Alpha, Delta, and Omicron, as the virus spread [42]. Thus, molecular diagnostics for SARS-CoV-2 span routine PCR testing for case confirmation to large-scale genomic surveillance of viral evolution [42].

8.1.4. H5N1 Avian influenza (A/H5N1)

Human infections with HPAI A(H5N1) are rare but often severe, making rapid detection essential. PCR-based influenza A assays have been adapted to include H5 subtyping, often in multiplex format. For instance, a single-step multiplex RT-PCR assay can simultaneously detect influenza A (**M gene**) and the H5 and N1 subtype genes in one reaction [43]. Such assays enable rapid confirmation of H5N1 in clinical specimens, typically respiratory swabs, during case investigations. WHO and CDC guidelines recommend RT-PCR targeting conserved influenza genes along with H5-specific probes to ensure prompt detection and reporting of novel zoonotic influenza strains [43]. **These molecular methods greatly reduce diagnostic time compared to virus culture and support rapid public health response when avian flu cases occur or if an H5N1 bird flu pandemic emerges** (Fig. 9).

Table 5
Comparison of LAMP and RPA isothermal amplification techniques.

Technology	Clinical Use / Scenario	Turnaround	Key Strengths	Limitations
Multiplex PCR	Rapid detection of common pathogens, critical results	1–4 h	Fast, sensitive, clinically validated	Limited to known targets
mNGS	Unknown/atypical pathogens, culture-negative infections	24–72 h	Unbiased, broad detection	Expensive, slower, requires bioinformatics
CRISPR assays	Rapid POC detection, AMR genes	<2 h	Ultra-sensitive, specific	Mostly experimental, limited multiplexing
LAMP	Decentralized/POC detection, resource-limited settings	30–60 min	Fast, simple, robust	Target-specific, lower throughput
WGS / WGS-AST	AMR profiling, outbreak tracing	24–72 h	Comprehensive genome info	Expensive, requires bioinformatics

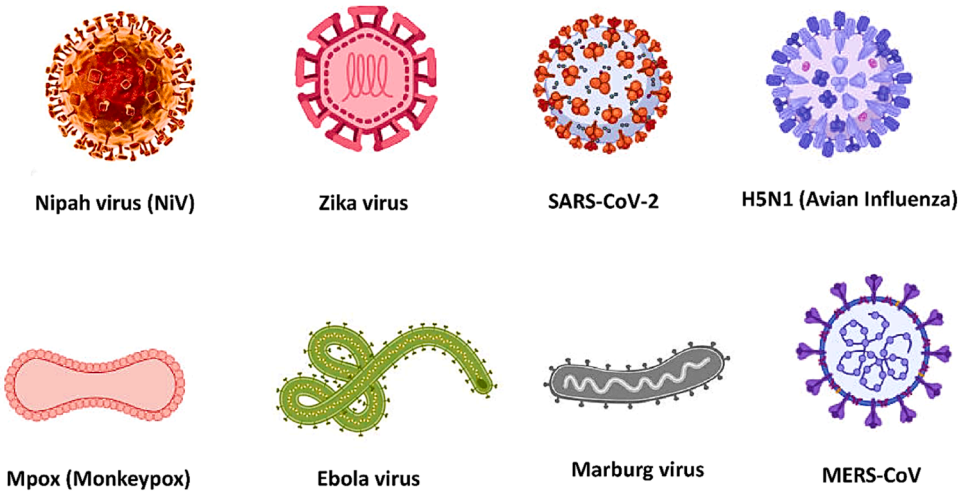

Fig. 9. Emerging viral pathogens of clinical and public health significance.

8.1.5. Mpox (monkeypox)

The 2022–23 global mpox outbreak underscored the need for reliable MPXV diagnostics. Molecular detection relies on PCR amplification of viral DNA from skin lesion material such as exudate, crusts, or swabs. During the outbreak, real-time PCR remained the reference standard for laboratory confirmation, and laboratory evaluations consistently demonstrated high diagnostic accuracy. Clinical studies, including those conducted in Italy, reported that multiple commercial and in-house assays (e.g., **Seegene** and **Altona Diagnostics**) achieved 96–100 % sensitivity and specificities up to 100 % in lesion-derived specimens, confirming the reliability of PCR-based detection of MPXV DNA [44, 45–52]. Accordingly, NAATs are used to confirm mpox cases definitively: individuals meeting the case definition are tested by PCR of lesion swabs, as recommended by WHO and CDC. These MPXV-specific primers do not amplify other pathogens, ensuring clear differentiation from other rash illnesses [44].

8.1.6. Ebola virus

In Ebola outbreaks (e.g., 2014–16 in West Africa and 2018–20 in the DRC), molecular testing has been indispensable. Confirmation of Ebola virus disease relies on laboratory diagnosis, with **RT-PCR on blood** as the standard method. A WHO review emphasizes that diagnosis "requires real-time reverse transcription PCR to detect viral RNA" in patient samples [53]. In practice, RT-PCR assays targeting Ebola virus genes such as **NP** or **L** are performed on **whole blood** or **serum** and yield results within hours. Although rapid antigen tests are available, they are less sensitive; PCR can detect infection early, before antibody responses appear. Consequently, in both clinical and field settings, a positive Ebola PCR result is considered definitive evidence of acute infection, enabling prompt isolation and contact tracing. PCR is also recommended for detecting Sudan and Bundibugyo viruses, the other epidemic filoviruses using analogous assays [53].

8.1.7. Marburg virus

Marburg virus disease is diagnosed similarly to Ebola. Because its symptoms resemble other hemorrhagic fevers, laboratory confirmation depends on PCR. As with Ebola, WHO guidelines state that "diagnosis requires real-time reverse transcription PCR to detect viral RNA" for filoviruses [53]. In outbreak settings, Marburg virus infection is confirmed by **RT-PCR of blood** or **tissue samples (liver, spleen, lymph nodes, kidney, lung, or skin biopsy)**. No rapid antigen tests are approved, making PCR the primary diagnostic method. After sample inactivation, RT-PCR assays often pan-filovirus formats can detect the Marburg virus genome within hours. The availability of high-quality molecular tests has greatly accelerated case identification compared to early outbreaks, such as in 1967 [53].

8.1.8. MERS-CoV

Middle East respiratory syndrome coronavirus (MERS-CoV), first identified in the **Arabian Peninsula** in **2012**, continues to cause sporadic infections and is regarded as a **betacoronavirus with concerning epidemic and pandemic potential**. Molecular testing is essential for diagnosis. Real-time RT-PCR assays targeting conserved coronavirus genes **upE, ORF1a/b**, and **N** remain the diagnostic standard [54]. WHO recommends initial screening for MERS-CoV RNA by RT-PCR of the upE gene, followed by confirmation using a second target such as ORF1a or N [54]. Thus, PCR functions as both screening and confirmatory testing. Reviews note that RT-PCR remains the "mainstay" of MERS-CoV diagnosis [54]. Viral culture or sequencing may be used for PCR-negative but clinically suspicious samples to detect divergent strains. Overall, molecular NAATs provide rapid confirmation of MERS cases and underpin laboratory surveillance in affected regions [54].

8.2. AMR surveillance

Molecular diagnostics have become important tools in tracking AMR. PCR-based assays (often multiplex) can directly detect known resistance genes or mutations in clinical samples or cultured bacteria (Fig. 10). For example, real-time PCR panels are used to screen isolates for carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, etc.) or methicillin-resistance gene mecA, providing results within hours. WGS of bacterial pathogens is increasingly used for AMR surveillance: WGS identifies all resistance determinants (including novel ones) and provides high-resolution strain typing. Public health labs now use WGS to monitor the emergence of resistant clones and plasmids (Fig. 10). Indeed, sequencing adds the ability to track genetic changes over time and geography, analogous to how viral sequencing tracks new strains [42]. By coupling targeted PCR screens with genomic analyses, health agencies can rapidly detect and catalog resistance patterns in hospital and community settings. These approaches greatly accelerate detection of AMR trends [55–59] compared to traditional phenotypic methods, although PCR-based tests still require updating as new resistance genes emerge.

8.3. Hospital outbreak investigations

Molecular diagnostics are essential for investigating hospital acquired infection clusters (Fig. 11). During an outbreak, rapid PCR assays can identify the pathogen and key resistance markers in patient samples and environmental swabs. Multiplex PCR panels can simultaneously detect multiple nosocomial bacteria or viruses in clinical isolates

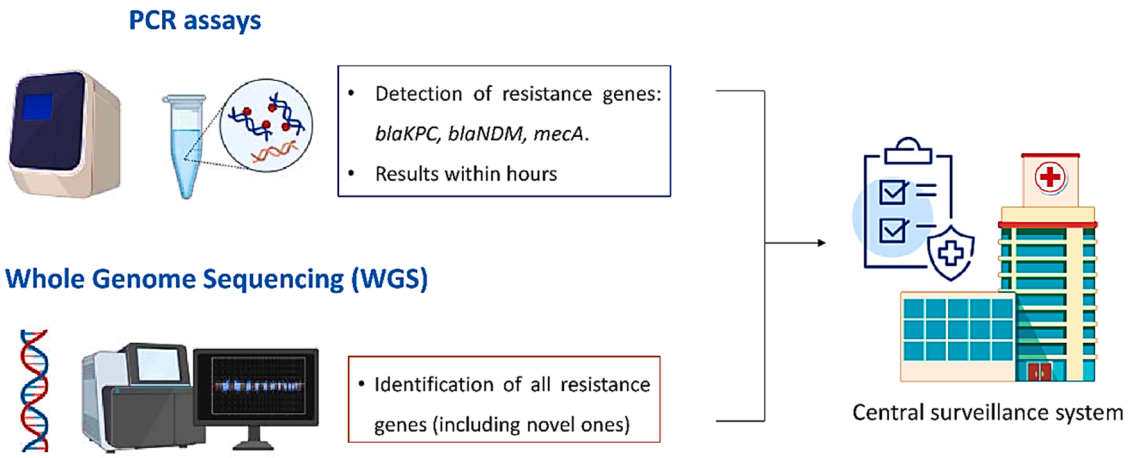

Fig. 10. Integration of PCR and WGS in AMR Surveillance.

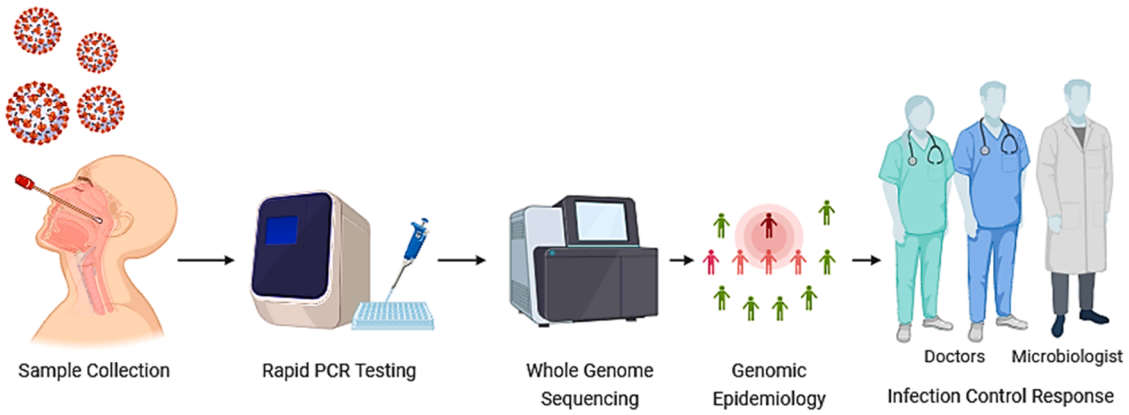

Fig. 11. Molecular approach to hospital outbreak detection and containment.

(Fig. 11). More commonly, WGS of isolates is used to establish transmission chains and strain relatedness. By comparing genomic sequences, epidemiologists, microbiologists, and clinicians can determine whether infections originate from a common source or represent distinct introductions. This genomic approach has proven highly effective in hospital outbreaks involving **MRSA, *C. difficile***, and carbapenem resistant **Enterobacterales** [55,56]. In short, PCR provides rapid pathogen identification, while sequencing supplies the genetic "fingerprint" needed to track spread. As one review noted for viral outbreaks, "the advantage of sequencing based detection is the ability to track mutations by gathering data on new strains" [42], a principle equally relevant to bacterial strain evolution. Integrated molecular testing, combining PCR and sequencing, thus enables infection control teams (Fig. 11) to pinpoint sources and transmission routes, facilitating swift outbreak containment [42].

9. Challenges and limitations

9.1. Cost and infrastructure requirements

Molecular diagnostic platforms require substantial upfront and recurring investment, as instruments such as real-time PCR systems and sequencers, along with proprietary reagents, remain costly [60]. Consumables, maintenance, calibration, and cold-chain requirements can exceed the operational capacity of small laboratories, particularly in low- and middle-income countries. A survey of African health facilities reported that financial constraints were the primary barrier to adopting molecular tests, with 26 % of facilities citing inadequate utilities such as unstable electricity and water supply as additional obstacles to routine implementation [61]. Beyond cost, molecular assays also necessitate dedicated laboratory space, biosafety infrastructure, and reliable power, restricting widespread deployment to well-resourced central laboratories and limiting access in peripheral or resource-limited settings.

In addition to these systemic barriers, each molecular technology has inherent limitations that influence diagnostic performance. mNGS faces challenges related to complex bioinformatics pipelines, absence of standardized workflows, and high host nucleic-acid background, all of which reduce sensitivity for low-abundance pathogens and increase cost and turnaround time; although strategies such as host depletion, targeted enrichment, and validated analytical pipelines improve performance, they do not fully resolve these limitations [62,63]. Detection of RNA viruses by mNGS and other DNA-centric workflows is also constrained by the intrinsic instability and lower abundance of RNA, necessitating optimized extraction, reverse transcription, and library preparation steps to achieve adequate sensitivity [64,65].

CRISPR-based diagnostics, while rapid and highly programmable, are restricted by sequence-context requirements (e.g., PAM/PFS motifs), potential off-target collateral activity, and limited multiplexing capacity. Although improved guide design and the use of orthogonal Cas enzymes can mitigate these issues, they do not eliminate concerns regarding cross-reactivity and assay fidelity [66,67]. Similarly, isothermal amplification platforms such as LAMP and RPA reduce instrument complexity but remain vulnerable to reagent instability and batch-to-batch variability when stored outside the cold chain. Advances in lyophilization and formulation have enhanced ambient stability, yet challenges persist for long-term use in decentralized or field

environments [68].

Multiplex PCR and syndromic panels broaden diagnostic coverage but are constrained by finite multiplexing capability, primer interactions, and inherent trade-offs between breadth and sensitivity for individual targets, requiring careful alignment of panel design with local epidemiology and clinical context [69]. Ultimately, across all platforms, additional hurdles including quality assurance, regulatory validation, and the need for robust clinical interpretive frameworks to distinguish true infection from colonization, contamination, or residual nucleic acid continue to slow translation into routine practice. Prospective outcome studies and standardized performance metrics are essential to link analytical improvements with meaningful clinical benefit [62,63].

9.2. Technical expertise and standardization issues

The operation and interpretation of molecular diagnostic assays demand skilled personnel, rigorous quality assurance (QA), and standardized procedures. However, a shortage of trained professionals and limited access to continuous education hinder consistent assay performance, particularly in developing regions. A recent pan-African meta-analysis found that clinical laboratories' average external quality assessment (EQA) performance was approximately 71 %, falling below the expected international benchmark of 80 % [70]. Similarly, a European interlaboratory study revealed significant variability in NGS outputs, with over 30 % of laboratories failing to adhere to standardized variant-calling and reporting guidelines [71]. Such discrepancies can lead to inconsistent or inaccurate results, compromising clinical decision-making and surveillance efforts. Standardized training, validation protocols, and accreditation systems are therefore critical to ensuring reliability, reproducibility, and comparability across molecular diagnostic platforms.

9.3. Sensitivity vs. specificity trade-offs

Molecular detection must be interpreted cautiously because highly sensitive assays can identify colonization rather than true infection. For example, multiplex respiratory panels frequently detect *Streptococcus pneumoniae* DNA in asymptomatic individuals; a meta-analysis of adults ≥60 years reported molecular carriage rates of 3–23 % [72], and other studies have documented upper airway colonization in 20–40 % of healthy individuals when PCR-based methods are used. This high prevalence of asymptomatic carriage underscores the need to correlate positive multiplex NAAT results with clinical symptoms, epidemiological context, and additional diagnostic findings. The superior sensitivity of nucleic acid amplification tests (NAATs) can also reduce clinical specificity by detecting non-viable organisms or commensals. In one comparative study, multiplex respiratory PCR panels identified bacterial DNA in culture-negative, asymptomatic patients, and 66.7 % of these detections were later determined to represent colonization rather than infection [73]. Moreover, nucleic acids may persist after pathogen clearance, leading to prolonged positive results even following effective therapy. Syndromic multiplex panels amplify these challenges by detecting multiple organisms simultaneously, some of which may not contribute to the patient's illness. Therefore, while molecular diagnostics provide exceptional analytical sensitivity, careful clinical correlation is essential to prevent overdiagnosis and inappropriate antimicrobial use [73].

Molecular diagnostics increasingly work alongside non–nucleic-acid-based technologies such as serology, MALDI-TOF proteomics, and emerging metaproteomic methods to provide a more comprehensive view of pathogen identity and host response. Serological assays remain essential for assessing immune history and status, and newer immunoaffinity-based proteomic techniques aim to improve the specificity and quantification of immunoglobulin isotypes and subclasses [74]. MALDI-TOF MS, now standard in many clinical microbiology laboratories, enables rapid peptide mass fingerprinting to identify bacteria, fungi, and some viral peptides, often reducing time to diagnosis by several hours. Integrative workflows that pair MALDI-TOF with PCR or sequencing support both species identification and strain-level surveillance; for example, MALDI-TOF-based detection of SARS-CoV-2 peptides has been achieved with high sensitivity and throughput. Metaproteomics extends these capabilities by characterizing the collective proteome of microbial communities and the host, linking genomic results to functional protein activity, including metabolic pathways, immune responses, and virulence mechanisms. Together, these multimodal approaches expand diagnostic breadth and depth, enabling a more complete understanding of infectious disease biology [75,76].

10. Future directions

10.1. Point-of-care molecular diagnostics

Recent years have seen the emergence of highly miniaturized NAAT platforms that bring laboratory-quality testing directly to bedside or field environments. Palm-sized RT-PCR devices, such as the FDA-approved Visby Medical unit, have demonstrated performance comparable to conventional systems, reporting around 95 % sensitivity and 100 % specificity [77]. Other innovations include fully self-contained microfluidic cartridges and portable high-throughput instruments. For example, the experimental MINI platform can run 96 parallel LAMP reactions in a rugged, hand-carried device [78]. These formats often powered by batteries, solar modules, or chemical heat pair rapid amplification with simplified workflows, supporting molecular diagnostics in clinics and remote settings without established laboratory infrastructure.

Despite this progress, many molecular POCT devices and their consumables remain costly and dependent on stable utilities and supply chains. Cold-chain needs, electricity instability, and limited funding significantly constrain their use in low- and middle-income countries, as highlighted in market surveys and programmatic assessments [79,80]. Moreover, while POCT can shorten turnaround times, affordability and operational robustness vary widely across platforms. Effective scale-up therefore requires coordinated procurement, maintenance systems, and integration into existing diagnostic networks rather than isolated deployment of individual devices [81].

10.2. Integration with AI and bioinformatics

Advanced computational tools are increasingly incorporated into molecular assays to improve data interpretation and predictive accuracy. Machine learning, for example, can now process complex multiplex PCR or high-resolution melt (HRM) outputs: a digital PCR–HRM system recently applied ML to deconvolute polymicrobial blood samples and identify bacterial species within four hours [82]. Deep learning applied to MALDI-TOF mass spectrometry similarly enables species-level identification directly from clinical samples and can even infer antimicrobial resistance profiles [82]. Sequencing workflows also benefit from AI-driven analytics real-time basecalling and classification pipelines, such as Oxford Nanopore's neural network basecaller and EPI2ME platform, assign taxonomic labels and variants as reads are generated, allowing near-immediate pathogen and mutation identification at the point of sequencing [83,84]. *In silico* assay design likewise uses data-driven models to optimize multiplex PCR or LAMP primer sets for improved coverage and reduced cross-reactivity. Collectively, these AI-enhanced bioinformatics tools are accelerating molecular diagnostics and improving interpretability by converting raw signal data into actionable identifications and resistance predictions in real time.

However, AI-enabled diagnostics also face significant constraints. Algorithmic bias from non-representative training data, limited external validation across settings, and poor model explainability can undermine generalizability and clinical confidence. Inadequate reporting standards further obscure data provenance and performance across relevant

subgroups. Addressing these issues requires prospective clinical validation, transparent and standardized reporting, and clinician-in-the-loop model designs [85,86]. Within a One Health framework, additional challenges arise: human, animal, and environmental data remain siloed by semantic heterogeneity, fragmented governance structures, and legal and ethical barriers to cross-sector sharing. Technical measures such as common metadata standards and interoperable APIs must therefore be paired with formal governance and trust frameworks to enable secure, timely, and actionable multisector data exchange [87,88].

10.3. Environmental surveillance and one health applications

Molecular diagnostics are increasingly central to One Health surveillance systems that integrate human, animal, and environmental health. Wastewater monitoring is a prominent example: routine PCR and NGS of sewage is now used globally to track pathogens such as **SARS-CoV-2** and **poliovirus**. Advanced analytics further enhance this approach; for instance, an unsupervised machine learning system applied to wastewater SARS-CoV-2 genomes detected the emergence of variants including **Delta, Omicron**, and **XBB** several weeks before they appeared in clinical sequencing data [89]. This **"one sample, many analyses" model** (Fig. 12) allows a single wastewater specimen to be tested by multiplex PCR for human viral pathogens, zoonotic influenza signatures, and antimicrobial resistance determinants, providing wide-ranging insights from a single environmental source [90].

Beyond wastewater, molecular tools are widely used in wildlife and livestock surveillance. Portable nucleic acid amplification tests support rapid field detection of **avian influenza in poultry flocks** and **arboviruses in mosquito pools**, directly linking animal health findings with public health decision-making (Fig. 13). Increasingly, portable nanopore sequencing is used to bridge human–animal genomic surveillance. Real-time nanopore sequencing on poultry farms including analysis of dust and environmental samples from H5-positive sites has enabled on-site detection of avian influenza virus (AIV) genomes and low-frequency variants, helping clarify viral transmission dynamics between avian species and mammals [91]. During AIV outbreaks in France (2020–2022), an amplicon-based nanopore sequencing workflow provided rapid genomic characterization of circulating H5 subtypes across affected farms [92]. Long-read nanopore sequencing has also been applied to paired human and avian influenza samples, identifying conserved targets such as PB2 and NS genes that can improve cross-species molecular diagnostics [93].

At the policy level, global One Health frameworks reinforce the integration of molecular and genomic surveillance. The Quadripartite Technical Group on Integrated Surveillance of Antimicrobial Use and Resistance (WHO, FAO, WOAH, UNEP) promotes harmonized cross-sector AMR monitoring, including genomic approaches across human, animal, and environmental reservoirs [94]. Likewise, the Tripartite Strategic Framework on AMR highlights whole-genome sequencing and molecular surveillance as essential components of coordinated One Health action [95].

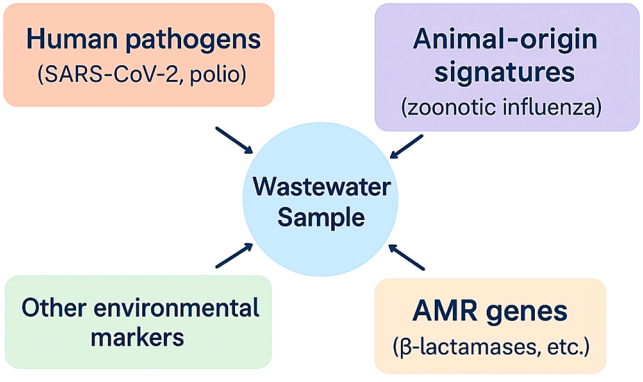

Fig. 12. One sample, many analyses: molecular outputs from wastewater surveillance.

Together, these scientific and policy advances demonstrate how molecular diagnostics and genomic tools now underpin One Health surveillance, enabling early detection of zoonotic threats such as H5N1, tracking of pathogen flow between species, and integrated monitoring of antimicrobial resistance. Such capabilities are critical for preemptive and coordinated responses to emerging infectious diseases.

11. Conclusion

Molecular diagnostics now constitute a central component of contemporary infectious disease management, providing rapid and highly sensitive detection that improves clinical decision-making, AMR surveillance, and outbreak control. Advances in PCR, isothermal amplification, digital PCR, NGS, and CRISPR-based detection have markedly enhanced diagnostic precision and turnaround time, and have been instrumental during recent emerging threats such as SARS-CoV-2, Mpox, and Nipah virus. Nonetheless, their widespread adoption remains limited by high costs, infrastructure needs, and the requirement for skilled personnel, particularly in resource-constrained settings.

The field is entering a pivotal phase in which incremental improvements will no longer be sufficient. Future progress will depend on the development of integrated diagnostic ecosystems that combine genomic, proteomic, serological, and host-response data with real-time bioinformatics and artificial intelligence. A key unresolved challenge is the integration of these heterogeneous data streams into clinically actionable frameworks capable of distinguishing infection from colonization, predicting disease severity, and supporting individualized antimicrobial therapy. Addressing this will require advances in computational modeling, standardized performance metrics, and robust multicentre clinical validation.

Embedding molecular diagnostics within One Health and environmental surveillance frameworks will further strengthen preparedness for zoonotic and environmental threats. Ultimately, the future of molecular diagnostics lies in scalable, equitable, and intelligence driven platforms that move beyond pathogen detection toward comprehensive, predictive, and preventive infectious disease medicine. Continued collaboration among clinicians, microbiologists, epidemiologists, and public health practitioners will be essential to fully realize this potential.

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

During the preparation of this work, the authors used Grammarly and QuillBot solely for language enhancement, grammar refinement, and improving readability. All AI-assisted outputs were carefully reviewed, fact-checked, and edited by the authors, who take full responsibility for the accuracy and integrity of the final manuscript.

Data availability

No data was used for the research described in the article.

CRediT authorship contribution statement

Sijo Asokan: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Avinash Choudekar:** Writing – review & editing, Resources, Formal analysis. **Arunkumar Jagadeesan:** Writing – review & editing, Resources,

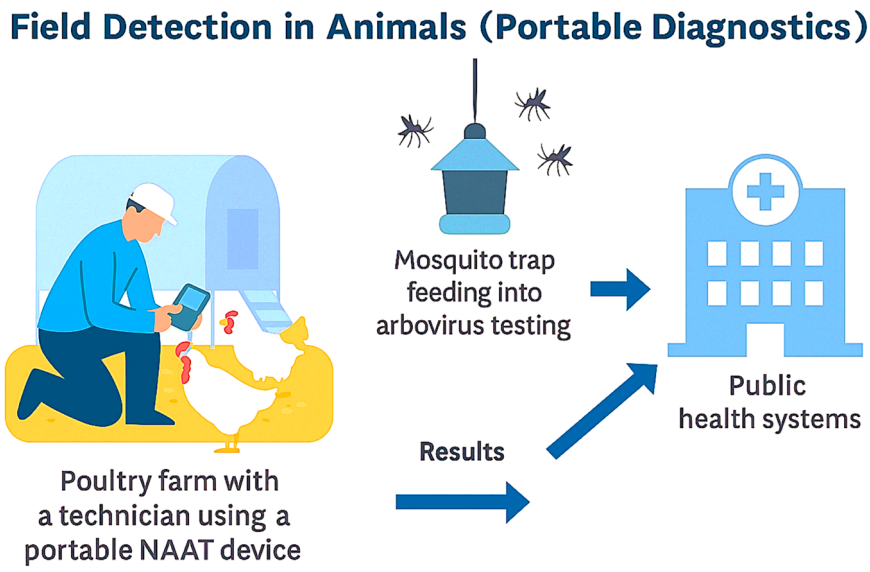

Fig. 13. Integrated field diagnostics for avian influenza and arbovirus surveillance.

Formal analysis. **Rudresh SM:** Resources, Investigation, Formal analysis. **Aamir Ali:** Resources, Investigation, Formal analysis. **Santosh Uttamrao Napte:** Resources, Formal analysis. **Saranya Ashokapuram Selvam:** Resources, Investigation, Formal analysis. **Gaurav Verma:** Resources, Investigation, Formal analysis. **Priti Das:** Resources, Formal analysis. **Nisha Beniwal:** Resources, Formal analysis. **Guhanraj Radhamanalan:** Resources, Formal analysis. **Smitha Vijayan:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Divya Rajeswary:** Resources, Investigation, Formal analysis. **Teena Jacob:** Data curation, Formal analysis, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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