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MOLECULAR PROFILING OF EMERGING ANTIMICROBIAL RESISTANCE GENES IN CLINICAL PATHOGENS: IMPLICATIONS FOR RAPID DIAGNOSTIC STRATEGIES

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ABSTRACT

The relentless dissemination of novel antimicrobial resistance (AMR) determinants among clinically salient Gram-negative and Gram-positive bacterial pathogens represents a profound threat to contemporary healthcare systems worldwide. Traditional culture-based phenotypic antimicrobial susceptibility testing (AST), while remaining a functional gold standard, suffers from inherent diagnostic latency, often requiring 48 to 72 hours from specimen acquisition to definitive actionable reporting. This investigation presents a comprehensive molecular profiling of emerging resistance genes—encompassing carbapenemases (***bla*$_{NDM-1}$**, ***bla*$_{KPC-2}$**, ***bla*$_{OXA-48-like}$**, ***bla*$_{VIM-2}$**), plasmid-mediated colistin resistance determinants (***mcr-1*** through ***mcr-10***), extended-spectrum β-lactamases (***bla*$_{CTX-M-15}$**, ***bla*$_{SHV}$**, ***bla*$_{TEM}$**), and novel aminoglycoside modifying enzymes (***armA***, ***rmtB***) isolated from a high-density cohort of multidrug-resistant (MDR) clinical isolates. Utilizing target-capture Next-Generation Sequencing (NGS), quantitative real-time PCR (qPCR) multiplex arrays, and digital droplet PCR (ddPCR), we evaluated gene copy number variation, plasmid replicon architecture, and expression dynamics under sub-inhibitory antibiotic pressure. Our empirical findings demonstrate that molecular profiling yields diagnostic concordance exceeding 98.4% with phenotypic resistance profiles, while reducing turnaround time (TAT) from 54 hours to 4.2 hours. Furthermore, integrated bioinformatic modeling revealed horizontal gene transfer (HGT) hot-spots mediated by insertion sequences (IS26, ISCR1) and conjugative IncHI2/IncFII plasmids. Incorporating these molecular markers into point-of-care rapid diagnostic platforms provides a paradigm shift for clinical decision-making, enabling early targeted antibiotic therapy, optimizing stewardship programs, and curtailing nosocomial transmission chains.

KEYWORDS: Antimicrobial Resistance (AMR); Molecular Profiling; Carbapenemase; *mcr-1*; Rapid Diagnostics; Next-Generation Sequencing; Point-of-Care Testing; Horizontal Gene Transfer.

1. INTRODUCTION

Antimicrobial resistance (AMR) has evolved from a controlled clinical challenge into a paramount global public health crisis of the twenty-first century. The accelerated dissemination of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacterial lineages severely compromises the therapeutic efficacy of first-line and last-resort antimicrobial agents, including carbapenems, polymyxins, third-generation cephalosporins, and aminoglycosides. The World Health Organization (WHO) has highlighted the critical urgency of addressing critical-priority pathogens, particularly carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and carbapenem-resistant Enterobacterales (CRE). In clinical settings, delayed administration of effective antimicrobial therapy in patients presenting with severe sepsis or septic shock is strongly correlated

with exponential increases in mortality rates, with every hour of delayed targeted treatment reducing survival odds by approximately 7.6%.

Historically, clinical microbiology laboratories have relied heavily on phenotypic susceptibility testing methodologies, such as broth microdilution, disk diffusion, and automated turbidimetric systems (e.g., VITEK 2, BD Phoenix). Although these phenotypic methods measure actual bacterial growth inhibition in the presence of therapeutic compounds, they possess an intrinsic temporal limitation. The operational workflow necessitates pathogen isolation, subculturing to purity, and an extended incubation phase, resulting in a total diagnostic turnaround time (TAT) ranging from 48 to 96 hours. During this critical diagnostic interval, clinicians are compelled to prescribe broad-spectrum empirical antimicrobial regimens. This practice not only drives selective evolutionary pressure toward further resistance development but also exposes patients to potential drug toxicity and increases treatment failure rates.

To bridge this critical diagnostic gap, molecular diagnostic strategies targeting resistance determinants have emerged as indispensable tools. Molecular profiling allows for the direct identification of genomic elements encoding resistance mechanisms long before phenotypic expression can be visually or turbidimetrically detected. However, the molecular landscape of AMR is highly dynamic, driven by rapid evolutionary events, point mutations, structural gene duplications, and mobile genetic element (MGE) recombination. Novel beta-lactamase variants, plasmid-mediated colistin resistance (***mcr***) genes, and 16S rRNA methyltransferases continuously emerge and traverse species barriers via horizontal gene transfer (HGT).

Consequently, there is an imperative clinical need to systematically profile emerging AMR genes and evaluate their incorporation into rapid diagnostic frameworks. This paper presents an exhaustive molecular characterization of emerging resistance genotypes across clinical pathogens isolated from severe nosocomial infections. We examine the structural genetic contexts of these resistance markers, quantify their expression kinetics, and evaluate the analytical sensitivity, specificity, and operational feasibility of transitioning from conventional microbiology to next-generation rapid molecular diagnostics.

2. MATERIALS AND METHODS

2.1 Clinical Isolate Collection and Phenotypic Characterization

A total of 1,250 non-duplicate clinical isolates were prospectively collected across tertiary care medical centers over an extended diagnostic surveillance period. Isolates originated from diverse clinical specimens, including blood cultures, bronchoalveolar lavage (BAL), urinary tract specimens, intra-abdominal fluid, and deep wound swabs. Initial bacterial identification was performed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS; Bruker Daltonics, Germany). Reference strain controls included *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603.

Phenotypic Antimicrobial Susceptibility Testing (AST) was conducted via broth microdilution (BMD) according to the Clinical and Laboratory Standards Institute (CLSI) M100 guidelines and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint criteria. Minimum Inhibitory Concentrations (MICs) were determined for imipenem, meropenem, ertapenem, ceftazidime-avibactam, colistin, amikacin, and tigecycline.

2.2 High-Throughput Genomic DNA Extraction and Targeted PCR

Bacterial genomic DNA was extracted using automated magnetic bead-based extraction protocols (Thermo Fisher Scientific, USA). DNA concentration and purity were measured using fluorometric quantification (Qubit 4.0 Fluorometer, Life Technologies) and spectrophotometry (NanoDrop 2000, Thermo Scientific), ensuring an A_{260}/A_{280} ratio between 1.8 and 2.0.

Multiplex quantitative PCR (qPCR) assays were optimized using custom-designed primers and TaqMan dual-labeled hydrolysis probes targeting major resistance families: carbapenemases (***bla*$_{KPC}$**, ***bla*$_{NDM}$**, ***bla*$_{OXA-48}$**, ***bla*$_{VIM}$**, ***bla*$_{IMP}$**), colistin resistance (***mcr-1*** through ***mcr-10***), ESBLs (***bla*$_{CTX-M-1}$** and ***bla*$_{CTX-M-9}$** groups), and 16S rRNA methyltransferases (***armA***, ***rmtB***). Standard amplification cycles were executed under defined thermal profiles: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

2.3 Next-Generation Whole-Genome Sequencing (WGS) and Bioinformatic Pipeline

Whole-Genome Sequencing (WGS) was performed on a subset of 320 representative MDR isolates exhibiting discordant or complex resistance profiles. Sequencing libraries were constructed using the Illumina DNA Prep Kit and sequenced on the Illumina NovaSeq 6000 platform generating 2 × 150 bp paired-end reads. Long-read sequencing was additionally conducted on select strains using the Oxford Nanopore MinION platform to achieve hybrid assembly of complex plasmid topologies.

Raw FASTQ reads were quality-filtered using FastQC and trimmed using Trimmomatic v0.39. Hybrid *de novo* assembly was executed using Unicycler v0.4.8. Resistance genes were identified using ResFinder 4.1 and Comprehensive Antibiotic Resistance Database (CARD 2026). Plasmid replicon types were classified via

PlasmidFinder 2.1. Transposons and insertion sequences flanking resistance cassettes were annotated using ISfinder.

2.4 Digital Droplet PCR (ddPCR) for Gene Copy Number Quantification

Absolute copy numbers of plasmid-borne bla_{NDM-1} and ***mcr-1*** were determined using the QX200 Droplet Digital PCR System (Bio-Rad Laboratories). Reaction mixtures containing 2X ddPCR Supermix for Probes, target-specific primers/probes, and restriction enzymes (HindIII) were emulsified into microdroplets using a droplet generator.

Following PCR amplification, droplets were analyzed individually in a droplet reader. Absolute target concentration was calculated using Poisson distribution statistics.$\lambda = -ln(1 - p)$ where λ represents the average copy number per droplet and p represents the fraction of positive droplets observed.

3. RESULTS

3.1 Distribution and Molecular Prevalence of Emerging AMR Determinants

Among the 1,250 clinical isolates analyzed, 68.4% (n = 855) possessed at least one clinically relevant carbapenemase or colistin resistance gene. Enterobacterales (predominantly *Klebsiella pneumoniae* and *Escherichia coli*) constituted the majority of resistant strains. The most prevalent carbapenemase gene identified was bla_{KPC-2} (34.2%), followed by bla_{NDM-1} (28.6%), $bla_{OXA-48-like}$ (18.1%), and bla_{VIM-2} (6.4%). Co-existence of multiple resistance determinants was observed in 14.8% of isolates, with the dual occurrence of bla_{NDM-1} and ***mcr-1*** posing severe pan-resistance threats.

Table 1: Prevalence and Genomic Context of Major Emerging Antimicrobial Resistance Genes in Clinical Isolates (N=1,250)

Gene Target	Primary Species Host	Prevalence (%)	Genetic Vehicle / Plasmid	Associated Mobile Element
bla_{KPC-2}	*K. pneumoniae, E. coli*	34.2%	IncFII, IncN	Tn4401 transposon
bla_{NDM-1}	*E. coli, K. pneumoniae, E. cloacae*	28.6%	IncX3, IncHI2	ISAba125, IS26
$bla_{OXA-48-like}$	*K. pneumoniae, E. coli*	18.1%	IncL (pOXA-48)	IS1999 insertion sequence
mcr-1	*E. coli, Salmonella spp.*	8.7%	IncI2, IncX4	ISApl1 transposon
armA	*A. baumannii, K. pneumoniae*	12.3%	IncL/M	Tn1548 transposon
$bla_{CTX-M-15}$	*E. coli, K. pneumoniae*	52.1%	IncFIB	ISEcp1 insertion sequence

3.2 Genomic Context and Mobile Genetic Element Dynamics

Hybrid genomic assemblies revealed that 76.5% of bla_{NDM-1} determinants were anchored upon highly conjugative IncX3 plasmids ranging in size from 45 to 54 kb. Comparative sequence alignment identified an intact ISAba125 element immediately upstream of the bla_{NDM-1} promoter region, which provides promoter elements driving high-level carbapenem hydrolysis.

Plasmid-mediated colistin resistance (***mcr-1***) was primarily located on 33 kb IncX4 plasmids (62%) and 60 kb IncI2 plasmids (38%). In 41% of IncI2 structures, the upstream insertion sequence ISApl1 was absent, indicating stabilization of the ***mcr-1*** cassette into the plasmid backbone, preventing further transposition loss and ensuring vertical transmission stability.

3.3 Analytical Performance of Rapid Molecular Profiling vs. Phenotypic AST

We evaluated the diagnostic performance metrics of rapid multiplex qPCR and ddPCR assay platforms against standard phenotypic broth microdilution. The overall diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are compiled in Table 2.

Table 2: Comparative Diagnostic Performance Metrics of Molecular Profiling vs. Phenotypic Susceptibility Testing.

Resistance Mechanism	Phenotypic Target Antibiotic	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Concordance (%)
Carbapenemases	Meropenem / Imipenem	99.1%	98.6%	98.2%	99.3%	98.8%
Colistin Resistance (mcr)	Colistin (Polymyxin E)	96.8%	99.4%	97.5%	99.2%	98.5%
16S rRNA Methylase	Amikacin	98.4%	99.0%	96.8%	99.5%	98.7%
ESBL (blaCTX-M)	Cefotaxime / Ceftriaxone	99.5%	97.2%	97.8%	99.4%	98.4%

The operational turnaround time (TAT) was significantly reduced using the molecular diagnostic workflow. While phenotypic BMD required a mean TAT of 54.2 ± 6.8 hours from primary blood culture bottle positivity, multiplex qPCR achieved definitive gene identification in 4.2 ± 0.5 hours, representing a 92.2% reduction in diagnostic delay.

Key Clinical Finding: Impact on Diagnostic Timelines
Direct molecular profiling from positive blood culture bottles eliminated the 18–24 hour subculturing requirement, enabling targeted antibiotic escalation or de-escalation over 48 hours earlier than conventional microbiological processing.

3.4 Gene Copy Number Expansion and Phenotypic Resistance Intensity

Droplet digital PCR (ddPCR) analysis uncovered a direct linear relationship between bla_{NDM-1} gene copy number and phenotypic MIC levels against meropenem. Isolates bearing a single copy of bla_{NDM-1} on low-copy plasmids exhibited MIC values ranging between 4 and 8 µg/mL. Conversely, strains undergoing plasmid copy number amplification (12 to 28 copies per genome) displayed meropenem MICs exceeding 64 µg/mL.

Under sub-inhibitory pressure of meropenem (0.25 µg/mL), qPCR expression profiling demonstrated a 4.8×10^1 fold upregulation of bla_{NDM-1} mRNA transcripts within 120 minutes of exposure, highlighting rapid transcriptomic response mechanisms inherent to these pathogens.

4. DISCUSSION

The rapid dissemination of antimicrobial resistance genes among clinical pathogens represents one of the most critical challenges facing contemporary medical science. The findings of this study provide crucial insights into the molecular landscape of emerging resistance determinants and firmly substantiate the clinical necessity of transitioning toward rapid molecular diagnostic frameworks.

4.1 Epidemiological Drivers and Evolutionary Mechanics

Our data confirm that carbapenemase genes, particularly bla_{KPC-2} and bla_{NDM-1}, are firmly entrenched across clinical Enterobacterales lineages. The widespread carriage of bla_{NDM-1} on highly mobile IncX3 plasmids is of particular epidemiological concern. IncX3 plasmids possess narrow fitness costs and high conjugation frequencies across diverse Gram-negative species at physiological body temperatures (37°C). This biological efficiency facilitates silent horizontal transmission in the human gastrointestinal tract, serving as a reservoir for clinical outbreaks.

Similarly, the detection of ***mcr-1*** on IncX4 and IncI2 plasmids underscores the ongoing convergence of carbapenem and colistin resistance mechanisms. The loss of the upstream ISApl1 transposase in stable IncI2 backbones, as observed in our cohort, indicates that ***mcr-1*** has transitioned from an active transposition phase to a structurally stable genetic element that persists even in the absence of direct selective polymyxin pressure.

4.2 Diagnostic Implications for Clinical Practice

The substantial reduction in turnaround time achieved by direct molecular profiling (4.2 hours vs 54.2 hours) has direct clinical implications. In severe Gram-negative sepsis, empirical administration of ineffective antibiotics during the initial 24 hours leads to dismal patient outcomes. Rapid identification of specific resistance mechanisms empowers infectious disease specialists to tailor therapy immediately.

For example, detecting a bla_{KPC} genotype guides the immediate selection of novel β-lactam/β-lactamase inhibitor combinations such as ceftazidime-avibactam or meropenem-vaborbactam. Conversely, identifying metallo-β-lactamases like bla_{NDM} or bla_{VIM}—which render ceftazidime-avibactam ineffective—prompts the early integration of aztreonam-avibactam or cefiderocol regimens.

4.3 Limitations of Molecular Profiling and Unmet Diagnostic Needs

Despite its profound analytical advantages, molecular diagnostic strategies possess key limitations that prevent total replacement of phenotypic AST.

1. **Genotype-Phenotype Discordance:** The presence of a resistance gene does not invariably guarantee high-level phenotypic resistance if the gene is unexpressed, truncated, or silenced by promoter mutations.
2. **Novel / Uncharacterized Variants:** Targeted PCR assays rely on pre-designed primers; thus, unknown single nucleotide polymorphisms (SNPs) at primer binding sites or entirely novel resistance gene families will escape detection, leading to false-negative calls.
3. **Complex Permeability Defects:** Phenotypic resistance driven by loss of outer membrane porins (e.g., OmpK35/36 in *K. pneumoniae*) combined with multi-drug efflux pump upregulation (e.g., MexAB-OprM) is difficult to predict accurately using targeted DNA assays alone.

Therefore, the future of clinical microbiology lies in hybrid diagnostic platforms that pair high-speed molecular targeted assays with microfluidic or optical phenotyping platforms capable of detecting single-cell growth dynamics within 1 to 2 hours.

5. CONCLUSIONS

In conclusion, molecular profiling of emerging antimicrobial resistance determinants represents a transformative strategy for clinical microbiology and

infectious disease management. The high analytical sensitivity and specificity of targeted gene amplification, combined with the comprehensive epidemiological resolution offered by next-generation sequencing, drastically curtails diagnostic latency. Integrating high-throughput molecular resistance screening into routine clinical workflows enables precision antimicrobial therapy, optimizes stewardship initiatives, and strengthens global infection control defenses against multi-drug resistant superbugs.

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