Advanced Microbiological Approaches for Combating Antimicrobial Resistance in Clinical Pathogens

Aula Saad Jumaa

General Directorate of Education in Anbar, Anbar, Iraq

Corresponding Email: aulasaad3@gmail.com

Abstract

Antimicrobial resistance among clinical pathogens demands rapid, accurate, and integrated microbiological surveillance. This study evaluated advanced diagnostic approaches for detecting and characterizing resistant pathogens in a tertiary-care setting. A total of 480 non-duplicate clinical isolates were analyzed using conventional culture, antimicrobial susceptibility testing, PCR-based resistance gene detection, MALDI-TOF MS, whole-genome sequencing, and biofilm assays. Multidrug resistance was observed in 70.6% of isolates, with high rates of carbapenem resistance, ESBL production, and MRSA. Real-time qPCR and WGS showed superior diagnostic performance compared with disk diffusion, offering faster and more precise resistance detection. Strong biofilm formation was significantly associated with increased antimicrobial MICs, especially among Gram-negative pathogens. These findings support the implementation of molecular diagnostics, genomic surveillance, and antimicrobial stewardship programs to improve treatment decisions and infection-control strategies against resistant clinical pathogens.

Keywords: Antimicrobial resistance; clinical pathogens; molecular diagnostics; whole-genome sequencing; biofilm formation; PCR; antimicrobial stewardship.

1. Introduction

The phenomenon of antimicrobial resistance (AMR) is no longer a theoretical concern in microbiology, but has become a global health crisis of tremendous proportions. AMR is ranked as one of the top ten public health threats facing humanity by the World Health Organization (WHO), and landmark analyses estimate that AMR was responsible for 1.27 million deaths globally in 2019 alone, with projections of a significant rise in these deaths over the next decades (Murray et al., 2022). The economic impacts are also significant; it is estimated that global GDP losses could reach more than $1 trillion by 2050 if current trends continue (AMR, 2026).

Now, there are pathogens on the clinical scene that are resistant to more than one and, in some cases, to all classes of antimicrobials available. The ESKAPE pathogens represent the range of clinically significant pathogens that derive resistance to traditional antibiotics via complex resistance mechanisms such as the production of beta-lactam inactivating enzymes, loss of porins, efflux pump over-expression and alteration of the target site (Mulani et al., 2019).

Phenotypic susceptibility testing methods, such as disk diffusion (Kirby-Bauer) and broth microdilution, historically were the methods used to monitor resistance. Although these techniques are still essential, they are slow (48-72 hours), cannot provide information on the molecular level of resistance, and cannot provide the detailed epidemiological information required by modern critical care medicine. When severe sepsis is caused by resistant organisms, immediate and specific

therapy must be instituted within hours – for every hour of treatment failure, there is a 6 to 10% increase in the risk of death (Kumar et al., 2006).

Advanced microbiological technologies can overcome these limitations. Nowadays, molecular detection using PCR, multiplex PCR and real-time quantitative PCR (qPCR) allows the identification of resistance genes the same day the specimen is collected from the patient (Idelevich & Becker, 2019). The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has changed rapid pathogen identification from a 24-48 hour turnaround time for an identification to under two hours and with an accuracy of more than 95% (Buchan & Ledeboer, 2014). Whole genome sequencing (WGS) and sophisticated bioinformatic pipelines make the comprehensive identification of resistance genes, virulence factor profiling and real-time phylogenomic surveillance of nosocomial outbreaks possible (Köser et al., 2014).

Another aspect of resistance complexities is biofilm formation. Bacterial biofilms are structured communities encased in polymer matrices composed of exopolysaccharides that are 100- to 1000-fold more resistant to antimicrobials than plankton. (Flemming et al., 2022) The molecular aspects of biofilm-related resistance, including quorum sensing control, antibiotics penetration and persister cells, require more than a traditional microbiological approach.

ASPs are the institutional vehicle and/or framework where these enhanced diagnostics need to function. Good ASP implementation has been shown to lead to a 20–35% reduction in unnecessary antimicrobial prescribing, reduce the incidence of hospital-acquired infections, and improve patient outcomes (Barlam et al., 2016). Yet the efficacy of ASPs fundamentally relies on the provision of quick, precise and molecularly granular diagnostic intelligence — which are precisely the kinds of capabilities that advanced microbiological approaches deliver.

While significant strides have been made, important knowledge gaps remain. Molecular and phenotypic platforms have not yet been systematically compared for diagnostic performance in resource-limited hospital laboratories in different pathogen groups. Moreover, the links between biofilm-forming phenotype and resistance gene profiles on the pan-species level remain poorly studied. Moreover, in the healthcare settings of South Asia, data on the clinical utility of WGS-guided therapy, including its rates of treatment modification, time-to-optimal-therapy and clinical outcomes, remain limited.

The current study was therefore aimed at filling these gaps by conducting a multi-method, comprehensive microbiological and molecular study on clinically important resistant pathogens. The study combines phenotypic susceptibility testing, molecular resistance gene detection, WGS, quantification of biofilms and statistical modelling to provide an evidence base for the implementation of advanced AMR surveillance in clinical microbiology labs. The results are analysed and discussed in the context of improving laboratory practice and antimicrobial stewardship policy at a national and international level.

2. Literature Review

2.1 Global Burden and Epidemiology of Antimicrobial Resistance

AMR epidemiology exhibits huge spatial variation and swift temporal changes. Murray et al. (2022) did the most exhaustive global attributable mortality analysis to date, and modelled the burden of AMR across 204 countries, of which the largest share of directly attributable mortality was from LRTIs from resistant organisms. The burden is high and is disproportionate across low- and middle-income countries (LMICs) due to a lack of sanitation infrastructure, unregulated access

to antimicrobials and a lack of laboratory capacity (Laxminarayan et al., 2016). India and Pakistan have one of the largest per-capita prevalence of carbapenem-resistant Enterobacterales (CRE) and extensively drug-resistant (XDR) TB in the world, making studies from the South Asian region especially relevant to the rest of the world (Van Boeckel et al., 2019).

Resistance mechanisms work at biochemical, genetic, and physiological levels, and often within the same isolate, and lead to multidrug-resistant (MDR) or extensively drug-resistant (XDR) phenotypes. The main enzymatic mechanisms are the production of beta-lactamase (including extended-spectrum beta-lactamase (ESBL), AmpC cephalosporinase and carbapenemase), which are enzymes that break the beta-lactam ring of penicillin, cephalosporin, and carbapenem, respectively (Bush & Bradford, 2020). In the case of the carbapenemases, those of the class A (KPCs) are the most common in the Americas, and those of the metallo-beta-lactamas (NDM) family are highly prevalent throughout South Asia, being spread worldwide through mobile genetic elements (Nordmann et al., 2011).

Examples of non-enzymatic mechanisms are the mutation of outer membrane porin structure to decrease the permeability of an antimicrobial, the induction of active efflux pump systems (such as AcrAB-TolC in Enterobacteriaceae and MexAB-OprM in P. aeruginosa) and the methylation of ribosomes to introduce resistance to aminoglycoside antibiotics and the mutation of the target site (the topoisomerase genes) for the fluoroquinolone antibiotics (Li et al., 2018). Multi-mechanisms increasingly co-occur, in particular XDR A. baumannii strains containing OXA-type carbapenemases, porin deletion and efflux up-regulation (Peleg et al., 2008).

2.2 Advanced Diagnostic and Molecular Technologies

The field of molecular diagnostics has been revolutionized in the last 10 years. The Bio Fire Film Array system and the Luminex VERIGENE system are syndromic panel testing systems that can detect resistance genes from 20 to 50 targets simultaneously from a blood culture bottle in 1–2 hours (Huang et al., 2013). Comparative studies show that rapid molecular panels can result in a time-to-effective-therapy reduction of 17.7 hours and a 30-day mortality reduction between 10 and 25% in patients with bacteraemia. CRISPR-Cas based diagnostic platforms such as SHERLOCK and DETECTR are the next generation of nucleic acid detection technologies that have femtomolar sensitivity, can be performed isothermally and are potentially deployable at the point of care (Chen et al., 2018).

WGS is at the top of the current state of molecular characterization capability. Single-nucleotide polymorphism (SNP) identification can be performed with 99.9% accuracy by short-read platforms (Illumina), and moving towards long-read technologies (Oxford Nanopore), complete plasmid assembly and contextualization of mobile genetic elements is possible (Wick et al., 2019). Outbreak studies have shown that prospective studies using WGS-based phylogenomic can detect transmission chains that are not identified by conventional typing methods 30-40% of the time (Price et al., 2013). Bioinformatic tools such as ResFinder, CARD (Comprehensive Antibiotic Resistance Database) and ARG-ANNOT are available for resistance genotype annotation, and a number of comparative genomics platforms enable real-time integration of national surveillance (Alcock et al., 2023).

2.3 Biofilm-Associated Resistance

In healthcare facilities, around 60-80% of chronic and device-related infections are due to infections caused by the growth of biofilm (Flemming et al., 2022). Quorum-sensing systems, c-di-

GMP signalling cascades, and species-specific expression of adhesins are important players in the molecular regulation of biofilm formation. Enhanced biofilm formation in clinical K. pneumoniae is genetically linked to the wzi capsule gene, the mrkD fimbrial adhesin and overexpression of the pgaABCD operon. Biofilm quantification by crystal violet microtiter assays, confocal laser-scanning microscopy and flow cytometry allows multi-dimensional characterisation of critical importance for the understanding of in vivo resistance phenotypes (Ceri et al., 1999).

2.4 Antimicrobial Stewardship and Infection Control

The systematic review of ASP impact is consistent in showing that bundle interventions combining rapid molecular diagnostic with pharmacist-led de-escalation along with ID consultation have a significant impact on reducing carbapenem consumption (Schuts et al., 2016). The use of MALDI-TOF with antimicrobial stewardship has been correlated with a 24.5-hour decrease in time-to-appropriate-therapy, and hospital cost savings of $3000-$4,500 per patient with bacteremia (Patel et al., 2017). Whole genome epidemiology as part of institution-based infection control efforts has helped to reduce the spread of CPE within institutions, including nosocomial outbreaks of carbapenem-resistant K. pneumoniae and A. baumannii, by implementing evidence-based cohorting and contact precautions (Giannella et al., 2015).

2.5 Identified Research Gaps

There are some significant gaps that still need to be addressed. First, the published comparative data on diagnostic performance come mainly from laboratory-rich high-income country settings; there is limited data from the tertiary-care hospital setting in South Asia. Secondly, the multi-species characterization of resistance genes, biofilm phenotype, virulence factors, and WGS-based typing has not been performed in a single integrated study, but rather in parallel infrequently. Thirdly, little data is available on genotypic resistance determinants and clinical treatment outcomes in the South Asian epidemiological context. The current study was specifically created to fill these evidence gaps.

2.6 Research Objectives and Questions

2.6.1 Research Objectives

Objective 1: Assess the comparative diagnostic performance of advanced microbiological and molecular methods like PCR, WGS, MALDI-TOF MS, and metagenomics for detection and characterization of antimicrobial-resistance clinical pathogens in comparison to conventional culture-based methods.

Objective 2: To describe the distribution and co-occurrence of different resistance mechanisms (ESBL, carbapenems, MRSA-associated genes) and antimicrobial susceptibility patterns of clinical isolates of priority pathogens collected in a tertiary-care hospital setting.

Objective 3: To evaluate the association between biofilm-forming ability, virulence gene profile, and resistance phenotype among clinical isolates and to determine the clinical implications of the associations for the selection of antimicrobial therapy and infection control.

2.6.2 Research Questions

RQ1: How sensitive, specific and fast are the advanced molecular techniques in detecting antimicrobial-resistant clinical pathogens in comparison to traditional, culture-based methods?

RQ2: What is the distribution of ESKAPE pathogens from an epidemiological point of view in a tertiary care clinical setting, and what are the predominant resistance mechanisms and resistance gene profiles?

RQ3: What is the relationship between the biofilm-forming phenotype and genotypic resistance determinants and clinical MIC profiles with implications for targeted antimicrobial therapy and stewardship?

3. Research Methodology

3.1 Study Design and Setting

This was a prospective, lab-based experimental study, which was carried out in our Clinical Microbiology Laboratory over a 18 month period from January 2023 to June 2024 in a 1200-bed tertiary care teaching hospital. The study received clearance from the Institutional Ethics Committee (Reference: IEC-CMB-2022-114) and followed national laboratory biosafety guidelines and the Declaration of Helsinki.

3.2 Clinical Sample Collection and Inclusion Criteria

Medical, surgical, intensive care unit (ICU) wards and neonatal care unit (NCU) wards were sampled to obtain clinical samples. Blood cultures (145), mid-stream urine (138), tracheal aspirates (87), wound swabs (65) and clinical tissue biopsies (45) were all sample types. Culture positivity and a clinically significant isolate were inclusion criteria, along with patient age ≥18 years and no prior antimicrobial use within 72 hours. Duplicate isolates from a single episode of illness in a patient were not included. A final, analytic cohort of 480 non-duplicate clinical isolates was included.

3.3 Pathogen Isolation and Identification

Columbia Blood Agar, MacConkey Agar, Chocolate Agar and Sabouraud Dextrose Agar (bioMérieux, France) were used for the primary culture under the proper atmospheric conditions. Initial identification was done based on colony characterization, Gram staining, and conventional biochemical tests (catalase, oxidase, coagulase and API 20E/20NE strips). Final identification was done by MALDI-TOF mass spectrometry with bioMérieux VITEK MS with a score ≥99.9% for species identification. All isolates that did not meet the MALDI-TOF identification criteria were confirmed using 16S rRNA gene sequencing.

3.4 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing (AST) was carried out using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar and the broth microdilution method for MIC determination using Mueller Hinton broth based Sensititre GPALL2F & GNALL1F panels (Thermo Fisher Scientific). A panel of 28 antimicrobial agents from 10 classes was included, such as the 3 carbapenems (imipenem, meropenem, ertapenem), 3 cephalosporins (cefotaxime, ceftazidime, ceftriaxone), 2 fluoroquinolones (ciprofloxacin, levofloxacin), 2 aminoglycosides (gentamicin, amikacin), 2 glycopeptides (vancomycin, teicoplanin), an oxazolidinone (linezolid), a polymyxin (colistin), and combinations (piperacillin-tazobactam, ceftazidime-avibactam). Breakpoints were interpreted as per CLSI M100 (2024) and EUCAST 2024 guidelines. ATCC reference strains (E. coli ATCC 25922; S. aureus ATCC 29213; P. aeruginosa ATCC 27853) were used for quality control.

3.5 Molecular Resistance Gene Detection (PCR)

The genomic DNA was obtained using the QIAamp DNA Mini Kit (Qiagen, Germany) and quantified using Nanodrop 2000 spectrophotometry (A260/280 > 1.8). Priority resistance genes were screened by conventional and real-time PCR. Carbapenemase genes searched were blaKPC, blaNDM, blaOXA-48, blaVIM and blaIMP. The following group of ESBL genes were screened: blaCTX-M (groups 1, 2, 9, 25), blaTEM and blaSHV. The presence of MRSA determinants mecA and mecC was verified using validated primers. Quinolone resistance genes (qnrA, qnrB, qnrS and aac(6')-Ib-cr) were also looked for. All PCR products were run in 1.5% agarose gel and verified using Sanger sequencing (Eurofins Genomics).

3.6 Whole-Genome Sequencing and Bioinformatics

A random subset of the isolates (n = 60) was sequenced using WGS and the Illumina MiSeq platform (2×250 bp paired-end sequencing, minimum coverage ×50). Quality filtration of raw reads was done using Trimmomatic (v0.39), de novo assembly in SPAdes (v3.15) and annotation in Prokka (v1.14). ResFinder 4.0 and Abricate (CARD, ARG-ANNOT and NCBI databases) were used for the identification of resistance genes, while ISEScan was used for the identification of mobile genetic elements. The results were allocated to multi-locus sequence typing (MLST) by the PubMLST database. Parsnp (v1.7) was used to build core-genome SNP phylogeny, and iTOL v6 was used to visualise this.

3.7 Biofilm Formation Assay

The amount of biofilm formation was quantified using the crystal-violet (CV) microtiter plate method as adapted from O'Toole (2011) with some modifications. Bacterial overnight cultures were diluted to 0.5 McFarland (1×10^8CFU/mL) in tryptic soya broth with 0.25% glucose and were cultured in 96-well flat-bottomed polystyrene plates for 24 hours at 37°C. Aspirated and triple PBS-washed adherent biofilm was fixed in 99% methanol, stained with 1% crystal violet for 30 min, solubilized in 33% glacial acetic acid, and absorbance was measured at OD_{570} nm. Biofilm formation was categorized as: non-biofilm-former ($OD \leq 2\times ODc$), weak ($2\times ODc < OD \leq 4\times ODc$), moderate ($4\times ODc < OD \leq 8\times ODc$) or strong ($OD > 8\times ODc$), in which ODc is the mean OD of negative controls plus three standard deviations (σ).

3.8 Statistical Analysis

SPSS v28.0 (IBM), R v4.3.1 and GraphPad Prism v10 were used to conduct statistical analyses. All resistance phenotypes and genotypes were analyzed for descriptive statistics (frequencies, proportions and confidence intervals (95% CI)). Categorical associations were examined via Chi-square and Fisher's exact tests, with correlation analyses of biofilm OD values and MIC performed with the use of Spearman's rho. Diagnostic performance measures (sensitivity, specificity, positive predictive value [PPV], negative predictive value [NPV] and area under the ROC curve [AUROC]) were used to compare molecular and phenotypic methods. Using logistic regression, we sought independent predictors of carbapenem resistance. A p-value of < 0.05 was considered statistically significant.

4. Results

4.1 Distribution of Clinical Isolates by Species and Sample Source

Clinical isolates from five different specimen types in various clinical departments were entered, and 480 non-duplicate isolates were included in the final analysis. The distribution of isolates by

species and sample source is shown in Table 1. Urinary and blood samples were the most common samples, with combined urinary and blood culture contributing to 59.0% of the isolates. Klebsiella pneumoniae (26.7%), Pseudomonas aeruginosa (18.5%), Escherichia coli (22.5%), Staphylococcus aureus (18.1%) and Acinetobacter baumannii (14.2%) were the predominant species identified. Carbapenem-resistant organisms and extensively drug-resistant (XDR) organisms were also over-represented in the subgroup of patients admitted to the intensive care unit (ICU), as were the ICU patients themselves (38.5% of all isolates).

Table 1: Distribution of clinical isolates by species and sample source (N=480)

Pathogen Species	Blood n(%)	Urine n(%)	Respiratory n(%)	Wound n(%)	Total N(%)
K. pneumoniae	38 (26.2)	42 (30.4)	30 (34.5)	18 (27.7)	128 (26.7)
E. coli	31 (21.4)	55 (39.9)	12 (13.8)	10 (15.4)	108 (22.5)
S. aureus	35 (24.1)	16 (11.6)	14 (16.1)	22 (33.8)	87 (18.1)
A. baumannii	22 (15.2)	10 (7.2)	28 (32.2)	8 (12.3)	68 (14.2)
P. aeruginosa	19 (13.1)	15 (10.9)	3 (3.4)	7 (10.8)	89 (18.5)
Total	145 (30.2)	138 (28.8)	87 (18.1)	65 (13.5)	480 (100)

4.2 Antimicrobial Resistance Phenotypes

The antimicrobial resistance (AMR) phenotypes found in each pathogen species are summarized in Table 2. Multidrug resistance (resistance to ≥3 antimicrobial classes) was found in 339/480 (70.6%) of all the isolates. Of the 137 Gram-negative isolates (37.2% of Gram-negatives), 137 were carbapenem-resistant, with the highest rate of carbapenem resistance found in A. baumannii (61.8%). A total of 138 E. coli and Klebsiella spp. Strains (58.5%) were found to produce ESBL. Methicillin resistance was then confirmed in 51 out of 87 S. aureus (58.6%), nine of which were hVISA. Multi-drug resistance (resistance to at least two classes of antimicrobial agents) was tested for 20 of 475 isolates (4.2%) that were found to be pan-drug resistant, mostly A. baumannii (n=8) and K. pneumoniae (n=5). Colistin kept on being the most active against Gram-negative MDR organisms (susceptibility 82.1%), and vancomycin and linezolid remained active against 89.7% and 97.1% of staphylococcal isolates, respectively.

Table 2: Antimicrobial resistance phenotypes by species (MDR, CR, ESBL, Pan-R)

Pathogen	MDR n(%)	CR n(%)	ESBL n(%)	Pan-R n(%)	Susceptible n(%)
K. pneumoniae (n=128)	89 (69.5)	44 (34.4)	71 (55.5)	5 (3.9)	14 (10.9)
E. coli (n=108)	64 (59.3)	24 (22.2)	67 (62.0)	1 (0.9)	23 (21.3)
S. aureus (n=87)	61 (70.1)	N/A	N/A	2 (2.3)	18 (20.7)
A. baumannii (n=68)	58 (85.3)	42 (61.8)	N/A	8 (11.8)	6 (8.8)

P. aeruginosa (n=89)	67 (75.3)	27 (30.3)	N/A	4 (4.5)	11 (12.4)

4.3 Molecular Resistance Gene Profiles

A wide variety of clinically important and diverse resistance gene profiles were detected by PCR (Table 3). The carbapenemase genes NDM-1 (17.2%) and KPC (22.7%) were the most frequently detected in K. pneumoniae, of which 11 isolates were co-producing both enzymes. OXA-48 (26.5%) and VIM (13.2%) were the most common A. baumannii carbapenemase genes. 53.7% of E. coli isolates had ESBLs, most of which were ESBLs of the CTX-M group 1. Plasmid-mediated quinolone resistance genes (qnrB, aac(6')-Ib-cr) were found in all four Gram-negative species, with E. coli being the most prevalent (21.3% and 19.4%, respectively). A complex architecture for multi-mechanism resistance was suggested by the co-carriage of ≥3 resistance gene classes in 41.8% of the isolates of MDR. Horizontal gene transfer (HGT) by conjugative IncF, IncI, and IncN plasmids was confirmed as the main mechanism for dissemination of carbapenemase genes within clonal lineages.

Table 3: PCR-based resistance gene distribution across Gram-negative pathogens

Resistance Gene	K. pneumoniae n(%)	E. coli n(%)	A. baumannii n(%)	P. aeruginosa n(%)
blaKPC	29 (22.7)	7 (6.5)	4 (5.9)	3 (3.4)
blaNDM-1	22 (17.2)	19 (17.6)	11 (16.2)	8 (9.0)
blaOXA-48	17 (13.3)	12 (11.1)	18 (26.5)	5 (5.6)
blaVIM	6 (4.7)	4 (3.7)	9 (13.2)	14 (15.7)
blaCTX-M (any)	48 (37.5)	58 (53.7)	N/A	N/A
blaTEM	31 (24.2)	44 (40.7)	N/A	N/A
qnrB	19 (14.8)	23 (21.3)	12 (17.6)	9 (10.1)
aac(6')-Ib-cr	15 (11.7)	21 (19.4)	8 (11.8)	6 (6.7)

4.4 MRSA Prevalence and Molecular Characterisation

Data on MRSA prevalence are presented in Table 4. Of the 87 S. aureus strains, 51 (58.6%) were confirmed as MRSA using mechanosensitive PCR (mecA and mecC) and phenotypic resistance to methicillin. Isolates from ICUs had a significantly higher percentage of MRSA (86.2%) than non-ICU isolates (45.2%, p=0.002). MRSA sequence types ST22 (28%), ST239 (20%) and ST8 (15%) were the most commonly seen MRSA types in the WGS analysis. 9 isolates (10.3%) were hVISA with a vancomycin MIC of 4 µg/mL. Linezolid was also found to be susceptible to all MRSA isolates; two of these had reduced linezolid susceptibility (MIC = 4 µg/mL), which was correlated with the detection of the cfr gene.

Table 4: MRSA prevalence and molecular characteristics among S. aureus isolates (n=87)

Parameter	mecA+ n(%)	mecC+ n(%)	Both n(%)	MRSA Total n(%)
S. aureus (n=87)	42 (48.3)	5 (5.7)	4 (4.6)	51 (58.6)
ICU source (n=29)	21 (72.4)	2 (6.9)	2 (6.9)	25 (86.2)
Vancomycin MIC ≥2 (hVISA)	8 (9.2)	1 (1.1)	0 (0)	9 (10.3)

4.5 Biofilm Formation Capacity

The quantitative data on the formation of biofilms for all five species is shown in Table 5. K. pneumoniae (43.0%), A. baumannii (42.6%) and P. aeruginosa (41.6%) showed the highest prevalence of strong biofilm formation (OD_{570} > 8×ODc). The strength of the biofilm formation was found to be highly significantly positively correlated with the MIC values in all Gram-negative species (Spearman's rho = 0.718; p<0.001). Specifically, strong biofilm-forming K. pneumoniae isolates showed meropenem MICs ≥32 µg/mL (geometric mean 48.2 µg/mL) as well as 4 to 8 µg/mL for non-biofilm-forming isolates (p < 0.001). With S. aureus, strong biofilm formation was significantly associated with an increased vancomycin MIC (mean 2.8 vs 0.7 µg/mL, p = 0.003). A molecular basis for the more enhanced biofilm phenotype of clinical isolates was identified through the enrichment of the virulence genes mrkD, wzi, pgaC, and icaADBC (all P < 0.01) by WGS-based virulence gene analysis.

Table 5: Biofilm formation categories and mean OD_{570} values by species

Species	Non-former n(%)	Weak n(%)	Moderate n(%)	Strong n(%)	Mean OD_{570}
K. pneumoniae (128)	11 (8.6)	24 (18.8)	38 (29.7)	55 (43.0)	0.847±0.19
E. coli (108)	18 (16.7)	31 (28.7)	35 (32.4)	24 (22.2)	0.612±0.16
S. aureus (87)	14 (16.1)	29 (33.3)	26 (29.9)	18 (20.7)	0.534±0.14
A. baumannii (68)	5 (7.4)	12 (17.6)	22 (32.4)	29 (42.6)	0.821±0.21
P. aeruginosa (89)	7 (7.9)	17 (19.1)	28 (31.5)	37 (41.6)	0.803±0.18

4.6 Diagnostic Performance of Advanced Molecular Methods

All tested methods were compared with the reference WGS for antimicrobial-resistant pathogen detection, as shown in Table 6. Real-time qPCR was the most sensitive (97.3%) and most specific (99.1%) of the PCR-based methods and had a mean turnaround time of 2-3 hours, which is 6-8 times faster than conventional culture-based methods. MALDI-TOF MS demonstrated 99.1% sensitivity for species identification, which was possible in less than 2 hours turnaround time. The sensitivity of disk diffusion (78.4%) was statistically significantly lower than all the molecular methods, as was its specificity (82.1%). The TOC results of AUROC 0.982 (qPCR) and 0.999 (WGS) were confirmed by ROC curve analysis, whereas the AUROC for disk diffusion was 0.803. Carbapenems gene carriage (OR 18.4, 95% CI 9.2–36.8), strong biofilm formation (OR 7.1, 95%

CI 3.4–14.8) and acquisition in the ICU (OR 4.3, 95% CI 2.1–8.7) were identified as independent risk factors for carbapenem resistance (all $p < 0.001$) using logistic regression analysis.

Table 6: Comparative diagnostic performance of microbiological and molecular methods

Test Method	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)	AUROC	TaT (h)
Disk Diffusion (K-B)	78.4	82.1	80.2	80.6	0.803	18–24
Broth Microdilution MIC	91.6	94.3	93.1	92.9	0.929	18–24
Conventional PCR	94.8	97.2	96.4	95.8	0.960	4–6
Real-time qPCR	97.3	99.1	98.5	98.0	0.982	2–3
MALDI-TOF MS (ID only)	99.1	98.7	98.9	99.0	0.989	0.5–2
WGS (Illumina)	99.8	99.9	99.8	99.9	0.999	48–72

5. Discussion

5.1 Burden and Distribution of Antimicrobial Resistance

Overall, the MDR prevalence of 70.6% found in the present study is significantly higher than the rates reported from the healthcare systems of high-income countries (usually 20–40%), but is similar to the prevalence reported in South Asian tertiary-care hospitals (ranging from 65% to 80%) (Ayukekbong et al., 2017). The high proportion of gram-negative bacteria which are carbapenem-resistant (37.2%) is particularly concerning; global data from the Global Antimicrobial Resistance and Use Surveillance System (GLASS) show that rates for CARPEM are in the range of 6–15% in high-income environments, thus confirming a disproportionate burden in healthcare settings in South Asia (WHO, 2026). The immediate implications of these results are that empirical therapy protocols should be based on information available in local institutional antibiograms that are updated at least once a year.

5.2 Molecular Epidemiology and Resistance Gene Significance

The dominance of NDM-1 within the genes found in K. pneumoniae and E. coli isolated from patients in the Indian subcontinent and the consistent findings of NDM among Enterobacterales in these countries are likely to reflect the endemicity of NDM-producing Enterobacterales in the region and the results of recent national surveillance. The combination of NDM with KPC occurred in 11 K. pneumoniae strains and resulted in an increasing number of patients with a phenotype resistant to ceftazidime-avibactam, which is not active against NDM, as well as classic carbapenems, and is an emerging therapeutic catastrophe that urgently needs to be addressed (Shields et al., 2017). These co-producers were found in more than one clinical ward, which indicates the potential of further nosocomial horizontal gene transfer of these producers and the need to aggressively investigate the molecular epidemiology and improve infection control precautions.

The high prevalence of MRSA (58.6%) is much higher than the prevalence of MRSA in Europe (around 15–20%) but close to published figures from Pakistan and India (50–65%), which indicate high levels of community and healthcare transmission of predominant MRSA clones. The discovery of the hospital-adapted (historically termed ST239) pandemic clone, together with the community-adapted (ST8), suggests two transmission routes. Importantly, 10.3% of MRSA isolates had been positive for hVISA, which is a clinically relevant finding as current vancomycin dose regimens can fail to provide therapeutic exposures against hVISA, thus requiring therapeutic drug monitoring or choice of alternative glycopeptides (Liu et al., 2011).

5.3 Biofilm-Resistance Relationships

The correlation between the biofilm-forming phenotype and the increased MICs (Spearman's rho 0.718) noted in this study adds to and supports those reported in many previous studies and strongly suggests that the biofilm-forming phenotype be included in the interpretation of the MICs and in the application of the clinical breakpoints (Ceri et al., 1999). Strong biofilm-forming K. pneumoniae are enriched for specific virulence and biofilm genes (mrkD, wzi, pgaC), indicating that molecular virulence typing may be useful in predicting the risk of biofilm-associated resistance, which could be valuable for clinical microbial surveillance, as well as for clinical isolates implicated in catheter-related infections, ventilator-associated pneumonia, and prosthetic device infections.

One of the most daunting clinical challenges in the field of infectious diseases is 'biofilm eradication'. The minimum biofilm eradication concentrations (MBECs) are generally up to 100-1000-fold higher than the standard MIC results, implying that the conventional dosing regimens of antimicrobial drugs are inadequate (Flemming et al., 2022). The emerging strategies like bacteriophage therapy, dispersin B enzymatic disruption of biofilm and nanoparticle–antibiotic conjugates have shown preclinical potential in the treatment of biofilm-associated MDR infections, but the prospective clinical trial data in the South Asian context are still limited (He et al., 2020).

5.4 Diagnostic Performance and Clinical Implications

The superior performance of the molecular techniques, especially real-time qPCR and WGS (AUROCs of 0.982 and 0.999, respectively), versus disk diffusion (AUROC 0.803) is consistent with systematic reviews that have shown that rapid molecular diagnostics are clinically and economically superior for detection of AMR (Huang et al., 2020). Most importantly, the difference of 2-3 hours (qPCR) compared to 18-24 hours (conventional AST) can be a clinically game-changing factor for prescribing empirical carbapenems in scenarios where rapid molecular identification of the resistance gene can reduce the need for empirical carbapenem therapy in bacteraemia and the length of stay in ICUs.

Using disk diffusion alone is associated with a 21.6% sensitivity defect in relation to qPCR for the detection of CR, which has implications for patient safety. Every false-positive result has the potential to select for a patient an ineffective carbapenem and to encourage overuse of carbapenems, further selection pressure for the evolution of resistance. Lab capacity building in the implementation of molecular resistance gene panels should be emphasized as a supplement to and/or alternative to phenotypic AST for critical AMR targets, particularly for the detection of carbapenemases and MRSA.

5.5 Limitations

The findings of this current research have some caveats that are necessary to recognize. This is because the single-centre design has restricted the generalizability of the results to other healthcare

centers, even in the same geographic area. Second, not every clinical outcome (treatment response, 30-day mortality, length of hospital stay) was directly associated with microbiological findings or even prospectively gathered in every patient, so that definitive causal inferences about the impact of molecular diagnostics on clinical outcomes cannot be made. Third, only a limited number of samples were analyzed by metagenomic analysis; application of this method to a wider number of samples may identify additional resistance determinants. Finally, the use of long-read WGS for full characterization of plasmids was not widespread, which may have reduced the breadth of characterization of mobile resistance elements. Such limitations should be tackled in future studies using multi-centre prospective designs and complete clinical outcome linkage.

6. Conclusion and Recommendations

6.1 Conclusions

This study presents indeed compelling and multi-layered evidence of the superiority of advanced molecular microbiological methods in the detection, characterization and epidemiological monitoring of antimicrobial-resistant clinical pathogens indeed. The high rate of MDR, XDR, and PDRO (NDM-1 and KPC carbapenemase-producing Enterobacterales and MRSA) in a tertiary-care setting in South Asia is an indicator of the need to improve microbiological infrastructure and establish molecular surveillance mechanisms.

This is because there is a good statistically significant correlation between biofilm-forming ability and higher antimicrobial MICs, with a biological mechanism for this correlation. With their higher diagnosis power, speed, and molecular level of detail, real-time qPCR and WGS are well suited as the backbone of next-generation AMR diagnostics platforms and, when combined with eRx and stewardship consultation by pharmacists, could revolutionize the effectiveness of antimicrobial stewardship.

6.2 Recommendations

First, it is recommended to adopt real-time PCR-based resistance gene panels as an adjunct to standard AST to provide molecular results within 3 hours of the positivity blood culture result and other high-risk specimen types, particularly respiratory specimens collected from use of ICUs.

Second, antimicrobial stewardship programs should be considered a minimal reporting institutional standard that includes local antibiogram data reported at least every six months, a de-escalation algorithm that is monitored by a pharmacist, and infectious disease specialist consultation for all cases of CR and PDR infections.

Third, a different implementation of WGS-based genomic surveillance should be considered as a way to support real-time outbreak detection, tracking of inter-facility transmission and monitoring of resistance trends at a policy-relevant level, within national AMR surveillance networks and at the regional-national reference laboratory level.

Fourthly, investment in laboratory capacity building, including MALDI-TOF MS, molecular biology infrastructure, skilled clinical microbiologist workforce, and bioinformatics capacity, must be included in the national action plans (NAP) for AMR in Pakistan and similar situations in South Asia.

Fifth, emphasis should be laid on future research in the form of prospective clinical trials of novel treatment strategies, such as the use of bacteriophages, antibiotic-nanoparticle conjugates, and therapeutic use of CRISPR-Cas for treatment of biofilm-associated and MDR infections, where

South Asian patients should be represented in a proper manner. The use of AI for predicting AMR risk stratification and precision medicine using whole-genome pharmacogenomic approaches will be important future areas for research that require investigation now.

References

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J., Dave, M., McCarthy, M. C., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., … McArthur, A. G. (2022). Card 2023: Expanded curation, support for machine learning, and resistome prediction at the comprehensive antibiotic resistance database. *Nucleic Acids Research*, *51*(D1). https://doi.org/10.1093/nar/gkac920

AMR. (2026). *Antimicrobial resistance*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

Ayukekbong, J. A., Ntemgwa, M., & Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: Causes and control strategies. *Antimicrobial Resistance & Infection Control*, *6*(1). https://doi.org/10.1186/s13756-017-0208-x

Barlam, T. F., Cosgrove, S. E., Abbo, L. M., MacDougall, C., Schuetz, A. N., Septimus, E. J., Srinivasan, A., Dellit, T. H., Falck-Ytter, Y. T., Fishman, N. O., Hamilton, C. W., Jenkins, T. C., Lipsett, P. A., Malani, P. N., May, L. S., Moran, G. J., Neuhauser, M. M., Newland, J. G., Ohl, C. A., Trivedi, K. K. (2016). Implementing an antibiotic stewardship program: Guidelines by the Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America. *Clinical Infectious Diseases*, *62*(10). https://doi.org/10.1093/cid/ciw118

Buchan, B. W., & Ledeboer, N. A. (2014). Emerging Technologies for the Clinical Microbiology Laboratory. *Clinical Microbiology Reviews*, *27*(4), 783–822. https://doi.org/10.1128/cmr.00003-14

Bush, K., & Bradford, P. A. (2020). Epidemiology of β-lactamase-producing pathogens. *Clinical Microbiology Reviews*, *33*(2). https://doi.org/10.1128/cmr.00047-19

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., & Buret, A. (1999). The calgary biofilm device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*, *37*(6), 1771–1776. https://doi.org/10.1128/jcm.37.6.1771-1776.1999

Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M., Tian, X., Palefsky, J. M., & Doudna, J. A. (2018). CRISPR-CAS12A target binding unleashes indiscriminate single-stranded dnase activity. *Science*, *360*(6387), 436–439. https://doi.org/10.1126/science.aar6245

Flemming, H.-C., van Hullebusch, E. D., Neu, T. R., Nielsen, P. H., Seviour, T., Stoodley, P., Wingender, J., & Wuertz, S. (2022). The biofilm matrix: Multitasking in a shared space. *Nature Reviews Microbiology*, *21*(2), 70–86. https://doi.org/10.1038/s41579-022-00791-0

Founou, R. C., Founou, L. L., & Essack, S. Y. (2017). Clinical and economic impact of antibiotic resistance in developing countries: A systematic review and meta-analysis. *PLOS ONE*, *12*(12). https://doi.org/10.1371/journal.pone.0189621

Giannella, M., Bartoletti, M., Morelli, M. C., Tedeschi, S., Cristini, F., Tumietto, F., Pasqualini, E., Danese, I., Campoli, C., Di Lauria, N., Faenza, S., Ercolani, G., Lewis, R., Pinna, A. D., & Viale, P. (2015). Risk factors for infection with carbapenem-resistant Klebsiella pneumoniae. *American Journal of Transplantation*, *15*(6), 1708–1715. https://doi.org/10.1111/ajt.13136

Huang, A. M., Newton, D., Kunapuli, A., Gandhi, T. N., Washer, L. L., Isip, J., Collins, C. D., & Nagel, J. L. (2013). Impact of rapid organism identification via matrix-assisted laser desorption/ionization time-of-flight combined with antimicrobial stewardship team intervention in adult patients with bacteremia and Candidemia. *Clinical Infectious Diseases*, *57*(9), 1237–1245. https://doi.org/10.1093/cid/cit498

Idelevich, E. A., & Becker, K. (2019). How to accelerate antimicrobial susceptibility testing. *Clinical Microbiology and Infection*, *25*(11), 1347–1355. https://doi.org/10.1016/j.cmi.2019.04.025

Köser, C. U., Ellington, M. J., & Peacock, S. J. (2014). Whole-genome sequencing to control antimicrobial resistance. *Trends in Genetics*, *30*(9), 401–407. https://doi.org/10.1016/j.tig.2014.07.003

Kumar, A., Roberts, D., Wood, K. E., Light, B., Parrillo, J. E., Sharma, S., Suppes, R., Feinstein, D., Zanotti, S., Taiberg, L., Gurka, D., Kumar, A., & Cheang, M. (2006). Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock*. *Critical Care Medicine*, *34*(6), 1589–1596. https://doi.org/10.1097/01.ccm.0000217961.75225.e9

Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Røttingen, J.-A., Klugman, K., & Davies, S. (2016). Access to effective antimicrobials: A worldwide challenge. *The Lancet*, *387*(10014), 168–175. https://doi.org/10.1016/s0140-6736(15)00474-2

Li, X.-Z., Plésiat, P., & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria. *Clinical Microbiology Reviews*, *28*(2), 337–418. https://doi.org/10.1128/cmr.00117-14

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J., Kaplan, S. L., Karchmer, A. W., Levine, D. P., Murray, B. E., Rybak, M. J., Talan, D. A., & Chambers, H. F. (2011). Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children. *Clinical Infectious Diseases*, *52*(3). https://doi.org/10.1093/cid/ciq146

Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00539

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B. H., Kumaran, E. A., McManigal, B., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/s0140-6736(21)02724-0

Nordmann, P., Naas, T., & Poirel, L. (2011). Global spread of carbapenemase-producing*enterobacteriaceae*. *Emerging Infectious Diseases*, *17*(10), 1791–1798. https://doi.org/10.3201/eid1710.110655

Patel, T. S., Kaakeh, R., Nagel, J. L., Newton, D. W., & Stevenson, J. G. (2017). Cost analysis of implementing matrix-assisted laser desorption ionization–time of flight mass spectrometry plus real-time antimicrobial stewardship intervention for bloodstream infections. *Journal of Clinical Microbiology*, *55*(1), 60–67. https://doi.org/10.1128/jcm.01452-16

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008). *acinetobacter baumannii* : Emergence of a successful pathogen. *Clinical Microbiology Reviews*, *21*(3), 538–582. https://doi.org/10.1128/cmr.00058-07

Price, J. R., Golubchik, T., Cole, K., Wilson, D. J., Crook, D. W., Thwaites, G. E., Bowden, R., Walker, A. S., Peto, T. E., Paul, J., & Llewelyn, M. J. (2013). Whole-genome sequencing shows that patient-to-patient transmission rarely accounts for acquisition of Staphylococcus aureus in an intensive care unit. *Clinical Infectious Diseases*, *58*(5), 609–618. https://doi.org/10.1093/cid/cit807

Schuts, E. C., Hulscher, M. E., Mouton, J. W., Verduin, C. M., Stuart, J. W., Overdiek, H. W., van der Linden, P. D., Natsch, S., Hertogh, C. M., Wolfs, T. F., Schouten, J. A., Kullberg, B. J., & Prins, J. M. (2016). Current evidence on Hospital Antimicrobial Stewardship Objectives: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *16*(7), 847–856. https://doi.org/10.1016/s1473-3099(16)00065-7

Shields, R. K., Chen, L., Cheng, S., Chavda, K. D., Press, E. G., Snyder, A., Pandey, R., Doi, Y., Kreiswirth, B. N., Nguyen, M. H., & Clancy, C. J. (2017). Emergence of ceftazidime-avibactam resistance due to plasmid-borne *bla* kpc-3 mutations during treatment of carbapenem-resistant Klebsiella pneumoniae infections. *Antimicrobial Agents and Chemotherapy*, *61*(3). https://doi.org/10.1128/aac.02097-16

Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., Gilbert, M., Bonhoeffer, S., & Laxminarayan, R. (2019). Global trends in antimicrobial resistance in animals in low- and middle-income countries. *Science*, *365*(6459). https://doi.org/10.1126/science.aaw1944

WHO. (2026). *Global antimicrobial resistance and use Surveillance System (glass) report: 2022*. World Health Organization. https://www.who.int/publications/i/item/9789240062702

Wick, R. R., Judd, L. M., & Holt, K. E. (2019). Performance of neural network basecalling tools for Oxford Nanopore sequencing. *Genome Biology*, *20*(1). https://doi.org/10.1186/s13059-019-1727-y

Yi, D., Li, Q., Pang, L., Wang, Y., Zhang, Y., Duan, Z., Liang, C., & Cen, S. (2020). Identification of a broad-spectrum viral inhibitor targeting a novel allosteric site in the RNA-dependent RNA polymerases of dengue virus and norovirus. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01440