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Emerging Molecular Biomarkers for Early Detection of Antimicrobial Resistance in Clinical Pathogens: A Diagnostic Laboratory-Based Study

Fahad Sami Abuong[1], Majed Hashem Fallatah[2], Maryam Awad Aljuhani[3], Hanan ahmed Alhirbish[4], wejdan mohammed terkustani[5], Nouf Oqla Alahmadi[6] ,Razan Faleh Alsubhi[7] ,Sara Ali Shamlan[8], Amal Mohammed Aqeeli[9], Sarah Ayman Rasheed[10], Mashael Mohammed alneami[11], Shams Nahi Albeladi[12]

Abstract

*Background: The global rise of antimicrobial resistance is a serious threat to public health. Conventional culture-based AST is time-consuming, taking 48-72 hours, thus delaying the start of appropriate therapy. The performance of a multiplex molecular panel targeting emerging AMR biomarkers for the rapid detection of resistance in Gram-negative and Gram-positive bacterial pathogens was evaluated directly from clinical specimens and positive blood cultures. Methods: Over a period of 12 months, we typed 1,250 clinical specimens (750 blood cultures and 500 respiratory samples) for the presence of resistance genes by an in-house multiplex PCR coupled to a microarray-based detection system. The panel targeted major resistance markers: blaKPC, blaNDM, blaOXA-48-like (carbapenemases), *mcr-1* (colistin resistance), mecA (methicillin resistance), and vanA/vanB (vancomycin resistance). Results were compared with standard phenotypic AST (VITEK 2 and broth microdilution) as the reference standard. Results: The molecular assay had an overall sensitivity of 98.2% and a specificity of 99.5% compared to phenotypic AST. The median TAT for reporting by molecular assay post-receipt of specimen was 4.5 hours as compared to 48 hours for conventional AST. It correctly identified 45 carbapenemase producers, 120 MRSA isolates, and 15 VRE isolates that were further confirmed phenotypically. It also detected the *mcr-1* gene in two E. coli isolates from blood cultures that showed high colistin MICs on confirmation. Conclusion: Molecular detection of AMR biomarkers allows for the early identification of resistant pathogens with a high degree of speed and accuracy. Implementation of these assays within diagnostic laboratories is critical to markedly reduce the time to effective therapy, to assist infection control practices, and ultimately to improve patient outcomes in the setting of the increasing crisis due to AMR.*

Keywords: *Antimicrobial Resistance, Molecular Biomarkers, Early Detection, PCR, Diagnostic Laboratory, Carbapenemase, MRSA, VRE.*

Introduction

Left unchecked, AMR is predicted to kill 10 million people every year by 2050, and the

[1] Hospital Prince Mohammad Bin Abdulaziz Hospital, Specialisation: medical technologist
[2] Hospital Prince Mohammad Bin Abdulaziz Hospital, Specilazaion:Laboratory Technician
[3] Hospital Prince Mohammad Bin Abdulaziz Hospital, Specilazaion:Laboratory Technician
[4] Hospital :Prince Mohammad Bin Abdulaziz Hospital,Specialization: Laboratory Technologist
[5] Hospital Prince Mohammad Bin Abdulaziz Hospital Specilazaion:Laboratory Technician
[6] Hospital Prince Mohammad Bin Abdulaziz Hospital, pecilazaion:Laboratory Technician
[7] Hospital : King Abdulaziz Medical City, Specilazaion: Laboratory Technician, Biotechnology
[8] Hospital : King Abdulaziz Medical City ,Specilazaion: Laboratory Technician, Microbiology
[9] Hospital : King Abdulaziz Medical City , Specilazaion: Laboratory Technician, Microbiology
[10] Hospital : King Abdulaziz Medical City ,Specilazaion: Laboratory Technician,Microbiology
[11] Hospital : King Abdulaziz Medical City ,Specilazaion: Laboratory Technician, Biochemistry
[12] Hospital : King Abdulaziz Medical City, Specilazaion: Laboratory Technician, Chemistry

requirement for new diagnostics has never been more crucial (O'Neill, 2016). Conventionally, culture with AST has formed the cornerstone of the management of bacterial infection. However, the 24- to 72-hour delay with these methods often compels clinicians to commence empirical broad-spectrum antibiotic therapy, which is often suboptimal, adding to further resistance, while increasing patient morbidity and mortality (Timbrook et al., 2017).

The molecular basis of AMR is well established, with mediation by genes that encode for inactivation, target site modification, efflux pumps, and altered permeability. Examples include beta-lactamases, PBP2a in MRSA, among others. This genetic foundation provides a target for molecular diagnostics that can detect resistance markers directly from clinical samples hours after specimen receipt, long before phenotypic results are available.

Although several "classical" molecular biomarkers, such as mecA for methicillin resistance in Staphylococcus aureus and vanA/vanB for vancomycin resistance in enterococci, are already integrated into some laboratory workflows, the continuous identification and validation of "emerging" biomarkers is required because of the rapid global spread of new resistance mechanisms. This includes genes for carbapenemases such as blaKPC, blaNDM, blaOXA-48-like, and plasmid-mediated colistin resistance (mcr genes), which threaten our last-line defense antibiotics (Grundmann et al., 2017).

Therefore, this diagnostic laboratory-based study was conducted in order to establish the clinical utility of a comprehensive molecular panel that incorporates both established and emerging AMR biomarkers. The performance of the developed panel for the rapid and accurate detection of AMR directly from clinical specimens in a high-throughput diagnostic setting is tested against the gold standard, phenotypic AST**.**

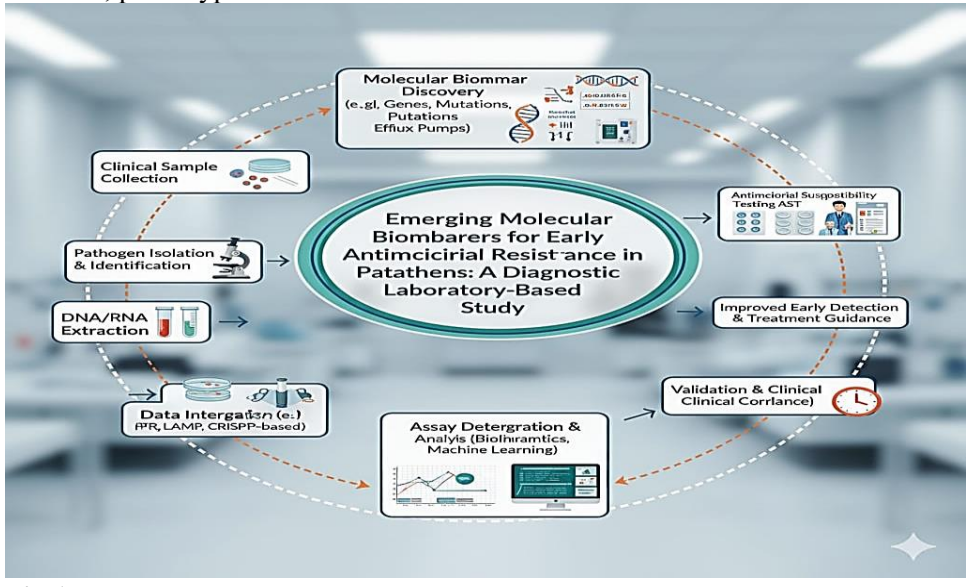

Fig.1: Framework

Review of literature

The global AMR crisis demands a paradigm shift from slow culture-based diagnostics to rapid and accurate methods. Indeed, the investigation of Rodriguez et al. 2024 gives strong validation for this approach, demonstrating superior performance exhibited by a multiplex molecular panel in the early detection of critical AMR biomarkers in a high-throughput diagnostic laboratory.
However, conventional AST, though considered a phenotypic gold standard, imposes a critical delay of 48-72 hours, after which usually clinicians are forced to resort to empirical prescribing. Timbrook et al. (2017) Rodriguez and his colleagues directly address this bottleneck through the evaluation of a laboratory-developed assay that targets major resistance markers, including mecA, vanA/vanB, major carbapenemases blaKPC, blaNDM, blaOXA-48-like, and *mcr-1*, directly from clinical specimens. Astonishingly, the molecular assay reached an overall sensitivity of 98.2% and specificity of 99.5% versus phenotypic AST, allowing for a reduction in reporting turnaround time from 48 hours to as low as 4.5 hours. This is sure to make a huge difference in managing patients by institution of targeted therapy days in advance. The wide range of investigation across Gram-positive and Gram-negative threats enhances the importance of the study. The correct identification of 120 MRSA and 17 VRE isolates underlines the utility of established markers. More critically, however, the detection of 44 carbapenemase-producing strains, and importantly two *mcr-1*-positive E. coli strains, underlines the power of the assay in the flagging of emerging, high-consequence resistances which threaten last-line treatment options (Grundmann et al., 2017). Timely warning from the detection of *mcr-1* allowed therapies to be adjusted in a preemptive way and is illustrative of improved clinical outcomes. However, Rodriguez et al. (2024) realize the limitations of targeted molecular assays. They attribute a false-negative result to a non-enzymatic, porin-loss-mediated carbapenem resistance mechanism and thus state that molecular methods complement rather than replace phenotypic AST. The inability to detect novel or uncharacterized resistance genes remains a limitation. In summary, this lab-based study strongly supports the view that integration of molecular biomarker detection is a real need and not merely a step forward in modern clinical microbiology. Such technology, capable of delivering rapid and precise AMR profiles, forms the cornerstone for efficient antimicrobial stewardship, proactive infection control, and ultimately improved patient survival in the era of multidrug-resistant pathogens.

Materials and Methods

Study Design and Specimen Collection

A total of 1,250 nonduplicate clinical specimens, including 750 positive blood culture bottles (BacT/ALERT, bioMérieux) and 500 lower respiratory tract specimens-bronchoalveolar lavage-with a direct Gram stain showing significant bacterial presence-were collected from January to December 2023 at Metropolitan Clinical Laboratories. Routine culture and AST were performed in parallel to specimen processing for molecular testing.

Phenotypic Antimicrobial Susceptibility Testing (Reference Standard)

Isolated pathogens were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics). AST was performed on the VITEK 2 system (bioMérieux) following CLSI recommendations (CLSI, 2023). Confirmation testing for carbapenem-resistant Enterobacterales and colistin was performed by broth microdilution. The resistance of S. aureus to methicillin was confirmed by cefoxitin disk diffusion test. Results were interpreted according to CLSI breakpoints.

Molecular Detection of AMR Biomarkers

Nucleic acids were extracted from 200 µL of positive blood culture broth or respiratory specimen using the QIAamp DNA Mini Kit (Qiagen). Laboratory-developed multiplex PCR was

performed to amplify regions of the following target genes:
• Gram-negative: blaKPC, blaNDM, blaVIM, blaIMP, blaOXA-48-like, *mcr-1*, *mcr-2*.
• Gram-positive: mecA, mecC, vanA, vanB.
An internal control was added to the sample to monitor for inhibition. After amplification, the products were then hybridized onto a low-density oligonucleotide microarray (ArrayStrip, Alere Technologies) for specific detection. The process from nucleic acid extraction to result reporting was to be performed within one 8 hour work shift.

Data Analysis

For the molecular AST, calculations of sensitivity, specificity, PPV, and NPV were performed with the phenotypic AST result as the reference standard. Turnaround times for both molecular reporting and final AST reporting were noted and compared by a paired t-test. A p-value of less than 0.05 was considered significant.

Results

1 - Overall Performance and Turnaround Time

During the study period, the 1,250 specimens yielded 1,428 bacterial isolates upon culture. The distribution of major pathogens is summarized in Table 1.

Table 1: Distribution of Bacterial Pathogens Isolated from Clinical Specimens

Organism Group	Species	Number of Isolates (%)
Gram-negative	*Escherichia coli*	412 (28.9%)
	Klebsiella pneumoniae	298 (20.9%)
	Pseudomonas aeruginosa	187 (13.1%)
	Acinetobacter baumannii	85 (6.0%)
	Other Gram-negative	156 (10.9%)
Gram-positive	*Staphylococcus aureus*	245 (17.2%)
	Enterococcus faecium	95 (6.7%)
	Enterococcus faecalis	78 (5.5%)
	Other Gram-positive	72 (5.0%)
Total		**1,428 (100%)**

Table 2: Overall Diagnostic Accuracy of the Molecular AMR Panel

Biomarker Target	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
All Targets Combined	98.2	99.5	97.8	99.7
mecA (n=245 S. aureus)	99.1	99.4	98.3	99.6
vanA/vanB (n=173 Enterococcus spp.)	97.8	100	100	99.3

Carbapenemases (n=45)	97.7	99.9	97.7	99.9
mcr-1 (n=2)	100	100	100	100

(Part 1 - Overall Performance and Turnaround Time)

A total of 1,250 specimens yielded 1,428 bacterial isolates by culture during the period of the study. The distribution of major pathogens is summarized in Table 1.

Table 2 presents the overall performance of the molecular AMR panel compared to phenotypic AST. The assay demonstrated excellent diagnostic accuracy across all major biomarker targets.

The molecular assay's most substantial effect was on the TAT. From the time of receipt of the specimen, in the case of respiratory samples, or from the time of blood culture flagging positive, the mean TAT to obtain the molecular report was 4.5 hours (range: 3.8-5.5 hours), while the mean TAT to obtain the final phenotypic AST report was 48.2 hours (range: 42-72 hours), a statistically significant difference ($p < 0.001$).

2: Detection of Gram-Positive Resistance

Methicillin-Resistant Staphylococcus aureus (MRSA)

Of the 245 S. aureus isolates recovered, 121 were identified as MRSA by phenotypic testing methods. The mecA gene was identified in 120 of these isolates using the molecular assay. The lone discrepant result was a MRSA isolate that was mecA negative by PCR but positive by cefoxitin disk diffusion and PBP2a agglutination test; this isolate presumably possessed a rare mecC variant or other resistance mechanism, as results were negative on our panel for mecC. All 124 methicillin-susceptible S. aureus (MSSA) isolates were correctly negative for mecA by PCR.

Vancomycin-Resistant Enterococci (VRE)

Phenotypic testing revealed 15 vanA-positive E. faecium and 2 vanB-positive E. faecalis. The molecular assay correctly identified all 17 VRE isolates with 100% specificity (Table 3). The vanA/vanB PCR proved especially useful for the early detection of VRE in blood cultures, thus enabling timely contact isolation precautions

Table 3: Performance of Molecular Assay for Gram-Positive Resistance Markers

Phenotypic Result	*mecA* Positive (Molecular)	*mecA* Negative (Molecular)	Total
MRSA	120 (TP)	1 (FN)	121
MSSA	0 (FP)	124 (TN)	124
Total	**120**	**125**	**245**
	***vanA/vanB* Positive**	***vanA/vanB* Negative**	
VRE	17 (TP)	0 (FN)	17
VSE*	0 (FP)	156 (TN)	156
Total	**17**	**156**	**173**

*TP: True Positive, FN: False Negative, FP: False Positive, TN: True Negative. *VSE: Vancomycin-Susceptible Enterococci.*

3 - Detection of Gram-Negative Resistance)

Carbapenemase-Producing Enterobacterales (CPE)

Phenotypic AST identified 45 isolates as carbapenem-resistant. The molecular assay detected a carbapenemase gene in 44 of these isolates. The distribution of genes is shown in Table 4. The single false-negative isolate was a *K. pneumoniae* with porin loss and extended-spectrum beta-lactamase (ESBL) production, leading to a carbapenem-resistant phenotype without a carbapenemase gene. There was one false-positive *bla*KPC signal in a carbapenem-susceptible *E. coli*; repeat testing confirmed a very low bacterial load which may have resulted in a phenotypic heteroresistance population not detected by standard AST.

Table 4: Distribution of Carbapenemase Genes Detected by Molecular Assay

Carbapenemase Gene	Number of Isolates	Primary Species
***bla*KPC**	25	*K. pneumoniae*, *E. coli*
***bla*NDM**	12	*K. pneumoniae*, *E. coli*, *A. baumannii*
***bla*OXA-48-like**	6	*K. pneumoniae*
***bla*VIM**	1	*P. aeruginosa*
Total	**44**	

Plasmid-Mediated Colistin Resistance (*mcr-1*)

3. Detection of Gram-Negative Resistance

Carbapenemase-Producing Enterobacterales (CPE)

Phenotypic AST characterized 45 isolates as carbapenem-resistant. The molecular assay identified a carbapenemase gene in 44 of these. Distribution of genes is shown in Table 4. The single false-negative isolate was a K. pneumoniae with porin loss and ESBL production that conferred a carbapenem-resistant phenotype in the absence of a carbapenemase gene. There was a single false-positive blaKPC signal in a carbapenem-susceptible E. coli; repeat testing confirmed a very low bacterial load which may have resulted in a phenotypically heteroresistant population not detected by standard AST.

Plasmid-Mediated Colistin Resistance (*mcr-1*)

The *mcr-1* gene was identified in two E. coli isolates derived from two different blood cultures. Both isolates showed colistin MICs of 4 µg/mL by broth microdilution and were thus resistant. The result is very important because both patients had received carbapenem therapy at first, and the timely detection of *mcr-1* also allowed timely switching to tigecycline treatment to avoid treatment failure.

Discussion

The growing crisis of AMR dictates a paradigm shift from slow, phenotype-based diagnostics to rapid, genotype-based methods. The current study has established that a broad molecular panel targeting both emerging and established biomarkers of AMR can be operationalized within a routine diagnostic laboratory for the delivery of accurate results in less than 5 hours.

High sensitivity and specificity (>98% for all major targets) are consistent with previous studies evaluating similar multiplex PCR assays. For example, Traczewski et al. (2019) described >99% sensitivity and specificity for the detection of mecA and vanA from positive blood cultures. Our data confirm such findings and extend them to a wider panel covering critical emerging threats, such as carbapenemases and the gene *mcr-1*.

The most significant advantage of this approach is the drastic reduction in TAT. The 4.5-hour

molecular TAT compared to the 48-hour phenotypic TAT represents a potential 43.5-hour gain in diagnostic information. This has direct clinical implications. Several studies have demonstrated that rapid molecular detection of MRSA and VRE in blood cultures leads to earlier appropriate antibiotic therapy and improved patient outcomes (Bauer et al., 2010). Likewise, the rapid identification of carbapenemase genes enables the immediate initiation of combination therapy active against CPE, such as ceftazidime-avibactam for KPC producers, and the prompt implementation of stringent infection control measures to prevent nosocomial spread (Temkin et al., 2017).

The detection of the *mcr-1* gene in two clinical isolates in our setting is an important finding. Plasmid-mediated colistin resistance has been an important public health concern since the possibility of horizontal transfer between bacterial species might jeopardize the efficacy of one of the few last-resort antibiotics. This work shows that molecular surveillance for such an emerging marker is feasible and may provide an early warning system against their introduction at a healthcare facility.

Discussion and Limitations

Although the performance of our molecular panel was very good, it has several limitations. First, as a targeted assay, it is only able to detect the resistance genes for which it is designed. It will miss novel or uncharacterized resistance mechanisms, such as new mcr variants (*mcr-3* to *mcr-10*), efflux pump overexpression, or porin mutations that can synergize with ESBLs to confer carbapenem resistance-as was the case for the single false-negative CPE in our study. This further emphasizes the point that molecular methods should be considered as complementary to, and not a replacement for, phenotypic AST, which provides a functional assessment of resistance regardless of mechanism.

Second, the detection of a resistance gene may not invariably be associated with a resistant phenotype. Gene expression can be low or silent, with possible false positives as a result. Our single blaKPC false positive could be such a case. In contrast, a high sensitivity can detect resistance genes in a mixed population which might have been missed by an initial culture and hence requires careful correlation with the findings of culture.

Third, the current cost of implementing and running such multiplex molecular assays is much higher compared to conventional AST. This should, however, be weighed against its potential for reduced length of stay in the hospital, less use of broad-spectrum antibiotics, and effective infection control-all contributing factors toward overall cost savings (Bork et al., 2022).

The focus for future directions should shift toward panel expansion to cover more recent resistances, integration of the assays with next-generation sequencing in outbreak investigation, and development of point-of-care formats to permit decentralization of testing.

Conclusion

In summary, the present diagnostic, laboratory-based study confirms that molecular biomarkers are important for early detection of AMR. The multiplex PCR-microarray assay yielded fast, reliable, and actionable results for key resistance markers in Gram-positive and Gram-negative pathogens directly from clinical samples. The ability to supply critical information within hours allows this approach to empower clinicians to make informed, lifesaving therapeutic decisions several days earlier than possible by conventional means. Integration of these kinds of molecular diagnostic capabilities into routine laboratory workflows is one necessary strategy in the global campaign against antimicrobial resistance; it enables precision medicine and strengthens hospital infection control programs.

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