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Molecular screening of quinolone resistance mutations in clinical *Acinetobacter baumannii* isolates using a mismatch amplification mutation assay PCR

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Abstract

Acinetobacter baumannii is a critical opportunistic pathogen known for its extensive antibiotic resistance. This study was conducted to characterise quinolone-resistant A. baumannii for key genetic markers. From a screening of 450 samples, a total of 32 isolates confirmed as A. baumannii by PCR targeting the bfmR gene. Seven of these isolates were found to be phenotypically resistant to quinolones and were selected for further molecular analysis. A three-primer mismatch amplification mutation assay (MAMA) PCR was employed to detect resistance-conferring mutations in the gyrA (S81L) and parC (S84L) genes. All seven isolates showed phenotypic resistance to ciprofloxacin, gatifloxacin and enrofloxacin. The MAMA-PCR results, validated by Sanger sequencing, indicated that six of the seven isolates harboured mutations in gyrA and five of these also possessed a mutation in parC, correlating with the observed phenotypic resistance. The MAMA-PCR provides a rapid and efficient method for screening genetic markers of resistance, which can be valuable for epidemiological surveillance.

Keywords: *Acinetobacter baumannii, MAMA-PCR, quinolone resistance, bfmR, gyrA, parC*

Acinetobacter baumannii has emerged globally as a significant Gram-negative coccobacillus responsible for a range of severe nosocomial infections (Peleg *et al.*, 2008). Its success as a pathogen is attributable to its intrinsic and acquired resistance to numerous antimicrobial agents and its remarkable ability to persist in the hospital environment (Lin and Lan, 2014). This has placed it within the ESKAPE group of pathogens that are a leading cause of hospital-acquired infections worldwide (Lee *et al.*, 2017). The non-prudent use of antibiotics in livestock production for therapeutic and prophylactic purposes is a contributing factor, leading to selection pressure for resistance and the potential for residues in foods of animal origin (Asif *et al*., 2020). The therapeutic utility of fluoroquinolones, a class of broad-spectrum antibiotics, has been severely compromised due to the high prevalence of resistance in *A. baumannii*. The primary mechanism of this resistance involves the accumulation of point mutations in the chromosomal genes *gyrA* and *parC*, which

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encode the subunits of DNA gyrase and topoisomerase IV, respectively (Hooper, 2001). These mutations reduce the binding affinity of quinolones to their target enzymes. The challenge of antimicrobial resistance necessitates the use of rapid molecular methods for investigation. Techniques such as mismatch amplification mutation assay (MAMA) PCR are powerful tools for detecting key genetic changes, like single nucleotide polymorphisms, in a rapid and cost-effective manner (Hamouda *et al*., 2010). Therefore, this study aimed to perform a preliminary molecular characterisation of quinolone-resistant clinical *A. baumannii* isolates to investigate the prevalence of key resistance determinants.

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

A total of 450 samples were initially collected and screened for the presence of *Acinetobacter* species. This screening yielded 32 isolates that were confirmed as *A. baumannii* via PCR targeting the *bfmR* gene. The isolates originated from diverse clinical, environmental, and hospital sources. Animal-derived clinical samples were collected from cattle (n=9), comprising nasal (n=6) and rectal swabs (n=3); dogs (n=8), comprising nasal (n=3) and rectal swabs (n=5); and goats (n=4), comprising nasal (n=3) and rectal swabs (n=1). Environmental isolates were obtained from soil (n=3) and water (n=5). Additionally, swabs from hospital contact surfaces (n=3) were collected from an examination table (n=2) and scissors (n=1).

Samples were processed for the selective isolation of *Acinetobacter* species using Leeds Acinetobacter Agar (Jawad *et al.*, 1994). The molten agar base was supplemented with an antibiotic solution containing vancomycin (10 mg), cefsulodin (15 mg) and cefradine (50 mg). Presumptive colonies were initially characterised through a panel of standard biochemical tests as described by Quinn *et al.* (2002) and Whitman *et al.* (2015). This included Gram staining to determine cell wall structure, followed by tests for oxidase and catalase activity. Further differentiation was performed using the IMViC (Indole, Methyl red, Vogues-Proskauer and Citrate) battery of tests, alongside a urease test to assess metabolic capabilities. A total of 32 isolates were confirmed as *A. baumannii* via PCR targeting the *bfmR* gene.

A subset of seven of these confirmed isolates that showed phenotypic quinolone resistance was selected for this detailed study. The isolates were cultured on MacConkey agar and nutrient agar to ensure purity. Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar and results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute for veterinary pathogens (CLSI, 2020) and for human medicine (CLSI, 2025). The isolates were tested against the quinolone antibiotics ciprofloxacin (CIP, 5 µg), gatifloxacin (GAT, 5 µg) and enrofloxacin (EX, 10 µg).

DNA extraction

Genomic DNA was extracted from overnight bacterial cultures of all seven isolates using a standard boiling lysis method (Sambrook and Russell, 2001). The concentration and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer before being stored at -20°C for use in subsequent PCR assays.

Molecular species confirmation

All isolates were subjected to PCR for species-specific confirmation by targeting the *bfmR* gene. The *bfmR* gene encodes a key response regulator that is part of a two-component system crucial for biofilm formation in *A. baumannii*. Due to its conserved nature and specificity, it serves as a reliable molecular marker for the accurate identification of this species (Tomaras *et al.*, 2008). The primers used for this reaction are detailed in Table 1. Each 25 µL PCR reaction mixture contained 1X PCR Master Mix, 0.4 µM of each forward and reverse primer, and approximately 50-100 ng of template DNA. The thermal cycling conditions are detailed in Table 2.

MAMA-PCR for gyrA and parC gene mutations

A three-primer MAMA-PCR system was used to screen for mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes (Hamouda *et al*., 2010). For each gene, a set of three primers was used: a common forward primer, an outer reverse primer and an inner reverse primer specific for the mutant allele. The common forward and outer reverse primers amplify a large fragment of the gene, which serves as an internal amplification control. The common forward and inner reverse primers are designed to amplify a smaller, mutation-specific product only when the mutation is present. The locations of the target amino acid substitutions were identified using the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al.*, 2023) and the inner reverse primer for each assay was designed to be complementary to the triplet codon encoding the specific substitution. In *A. baumannii*, a Serine-to-Leucine substitution was targeted at codon 81 of *gyrA* (S81L) and at codon 84 of *parC* (S84L), mutations which have been previously associated with quinolone resistance (Hujer *et al.*, 2009), yielding a 152 bp and 227 bp product, respectively. The primers used in this study for the *gyrA* (S81L) and *parC* (S84L) assays were designed using the Primer-BLAST tool from NCBI and their expected amplicon sizes are detailed in Table 1. The MAMA-PCRs were performed in a 25 µL reaction volume containing 1X PCR Master Mix and approximately 50-100 ng of template DNA. The primer mixture was specifically formulated to contain 0.4 µM of the common forward primer and 0.2

Table 1. Details of primers used for PCR and MAMA-PCR assays

Target Gene	Primer Name	Primer Sequence (5' - 3')	Amplicon Size (bp)	Reference
bfmR	bfmR-F	5'-GCGATAAAATACGGCCAGCG-3'	424	Tomaras *et al.* (2008)
	bfmR-R	5'-GGTAACCGTGCAATTCGTCG-3'		
gyrA (S81L)	gyrA-F (Common)	5'-ATTGCCGGATGTGAGAGACG-3'	847 (Outer)	This study
	gyrA-R (Outer)	5'-CGACTTCTGCGTTTTCACCG-3'		
	gyrA-R (Inner-Mutant)	5'-GCTAAGTCACCATGCGGGT-3'	152 (Inner)	
parC (S84L)	parC-F (Common)	5'-AAAACCGCTCTGTAGCCGAA-3'	598 (Outer)	This study
	parC-R (Outer)	5'-TTTTTCGTCCGAGGTTTGCG-3'		
	parC-R (Inner-Mutant)	5'-ATGCCAAGTCACCATGTGGG-3'	227 (Inner)	

Table 2. Thermal cycling conditions for PCR assays

Step	*bfmR* PCR	MAMA-PCR (*gyrA*)	MAMA-PCR (*parC*)
Initial Denaturation	95°C for 5 min	95°C for 5 min	95°C for 5 min
35 Cycles			
Denaturation	95°C for 45 s	94°C for 45 s	95°C for 45 s
Annealing	58.3°C for 1 min	59.2°C for 1 min	58.6°C for 1 min
Extension	72°C for 1 min	72°C for 1 min	72°C for 1 min
Final Extension	72°C for 10 min	72°C for 10 min	72°C for 10 min
Hold	4°C	4°C	4°C

µM each of the inner mutant-specific and outer reverse primers. The specific thermal cycling conditions used for the *gyrA* and *parC* MAMA-PCR assays are detailed in Table 2.

Sanger sequencing validation

To validate the MAMA-PCR results, representative isolates positive for the *gyrA* and *parC* mutations were selected for confirmatory sequencing, along with a wild-type control. The QRDRs of the respective genes were amplified using the outer primer pairs. The resulting amplicons were purified and subjected to bidirectional Sanger sequencing. Sequence analysis was performed using MEGA12 software by aligning the data with *A. baumannii* reference sequences from NCBI to confirm the specific nucleotide substitutions.

Results and discussion

The increasing incidence of infections caused by multidrug-resistant *A. baumannii* presents a significant therapeutic dilemma in both human and veterinary medicine. The clinical manifestations of *A. baumannii* infection are diverse, ranging from pneumonia and bacteraemia to wound infections, often complicated by the organism's robust biofilm-forming capabilities (Munoz-Price and Weinstein, 2008). In this study, isolates showed characteristic growth on selective and differential media, appearing as non-lactose fermenting colonies on MacConkey agar and growing well on the selective Leeds Acinetobacter Agar (Fig. 1). All seven clinical isolates selected for analysis exhibited phenotypic resistance to all three tested fluoroquinolones, showing no zones

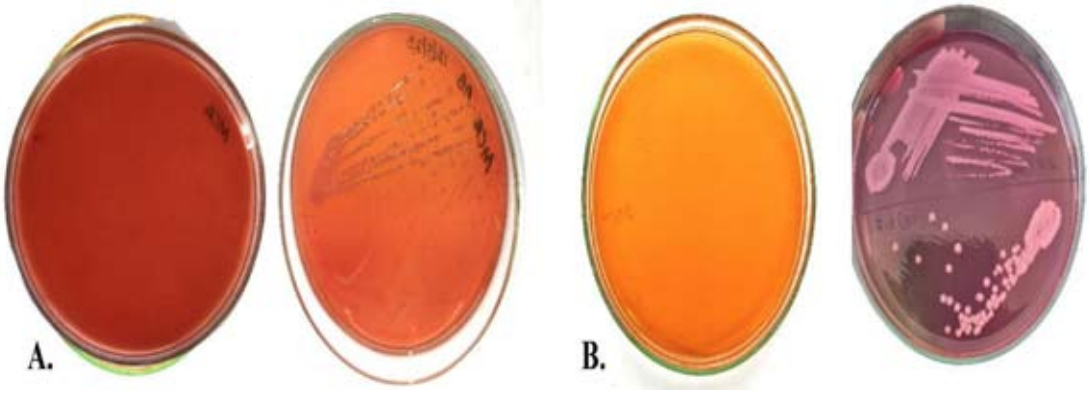

Fig. 1. Cultural characteristics of *A. baumannii*. (A) Non-lactose fermenting colonies on MacConkey agar (left-uninoculated, right-colourless colonies). (B) Growth on selective Leeds Acinetobacter Agar (left-uninoculated, pinkish white-colour colonies).

Fig. 2. Antimicrobial susceptibility testing on Mueller-Hinton agar. The image shows the resistance profile of a representative *A. baumannii* isolate. The absence of zones of inhibition around the antibiotic disks indicates resistance to ciprofloxacin (CIP, 5 µg), gatifloxacin (GAT, 5 µg) and enrofloxacin (EX, 10 µg). The plate also shows susceptibility to imipenem (IPM, 10 µg), which was used as a control antibiotic.

of inhibition around the ciprofloxacin, gatifloxacin and enrofloxacin disks on Mueller-Hinton agar (Fig. 2). This indicated a high level of quinolone resistance within this sample group.

Molecular confirmation is crucial for accurate identification. All seven isolates (100%) yielded the expected 424 bp amplicon for the *bfmR* gene, confirming them as *A. baumannii* (Fig. 3).

The MAMA-PCR was employed to rapidly screen for mutations in *gyrA* and *parC*. The results were validated by Sanger sequencing, which confirmed the accuracy of the assay. The results of the *gyrA* MAMA-PCR showed that six of the seven isolates (85.7%) produced both the 847 bp outer control band and the 152 bp inner band corresponding to the S81L mutant allele. Sequencing confirmed the S81L substitution (TCA > TTA) in the mutant isolates (Fig. 4). The mutation in the *gyrA* gene is commonly reported as a reason for quinolone resistance (Ashtamy *et al*., 2023).

For the *parC* gene, the MAMA-PCR revealed that five of the seven isolates (71.4%) amplified both the 598 bp outer control band and the 227 bp inner band indicative of the S84L mutant allele. This corresponds to a TCG to TTG nucleotide change, resulting in the S84L substitution, which was also confirmed by sequencing (Fig. 5). All five isolates positive for the *parC* mutation were also positive for the *gyrA* mutation. This finding is consistent with previous reports that a *gyrA* mutation is often the primary event in developing quinolone resistance, with a subsequent *parC* mutation leading to higher levels of resistance (Vila *et al.*, 1996; Perez *et al.*, 2007). The presence of these mutations in the majority of the phenotypically resistant isolates demonstrates a good correlation between genotype and phenotype. Companion animals like dogs are considered potential carriers of microbes with AMR genes to human beings (Ashtamy *et al*., 2023). Furthermore, the detection of resistant isolates from soil is noteworthy, as soil can act as a significant environmental reservoir for opportunistic pathogens, potentially impacting environmental, animal and human health systems (Ananya *et al*., 2025).

The isolation of quinolone-resistant *A. baumannii* from animal sources in this study underscores the need for prudent use of antibiotics in livestock and a robust monitoring system to mitigate public health risks (Asif *et al*., 2020). The use of MAMA-PCR in this study proved to be a simple, rapid and effective tool for screening genetic resistance determinants. The accuracy of the assay was confirmed through Sanger sequencing of representative isolates, which verified that the mutation-specific primers were amplifying the correct alleles. This validation supports its utility as a reliable screening tool for epidemiological tracking of known resistance mutations.

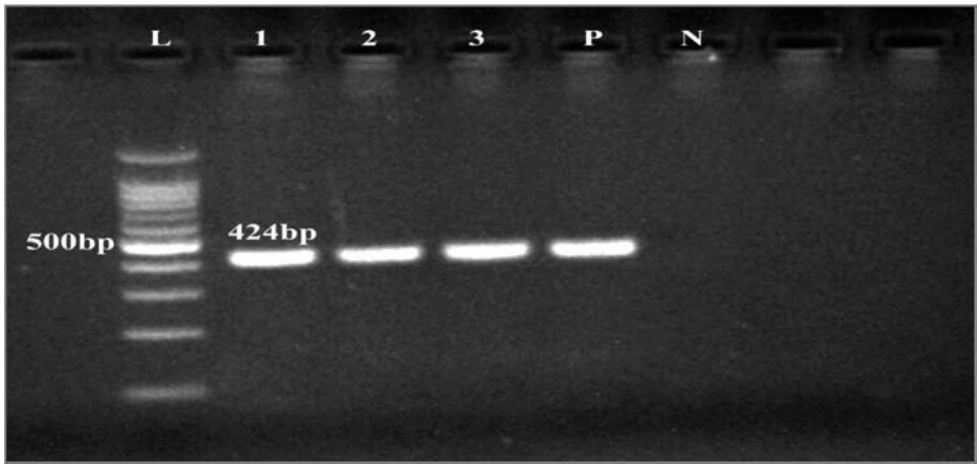

Fig. 3. Agarose gel (1.5%) showing amplification products for the *bfmR* gene. Lane L: 100 bp DNA ladder; Lanes 1-3: Test isolates showing positive amplification of the 424 bp *bfmR* product; Lane P: Positive control; Lane N: Negative control.

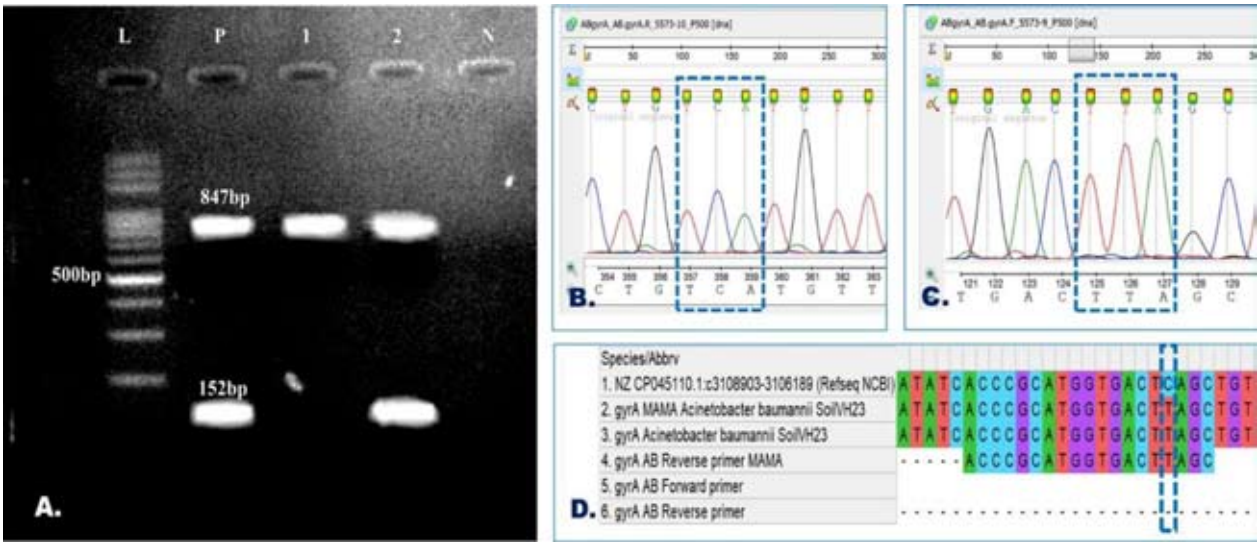

Fig. 4. Validation of *gyrA* (S81L) MAMA-PCR. (A) Representative agarose gel (2%) of MAMA-PCR results. Lane L: 100 bp DNA ladder; Lane P: Positive control (mutant); Lane 1: Test isolate negative for S81L mutation (only 847 bp band, wild type); Lane 2: Test isolate positive for S81L mutation (847 bp and 152 bp bands- mutant); Lane N: Negative control. (B) Sanger chromatogram of the wild-type isolate, showing the TCA codon. (C) Chromatogram of the mutant isolate, confirming the TTA codon. (D) Multiple sequence alignment showing the single nucleotide polymorphism (MEGA12 software).

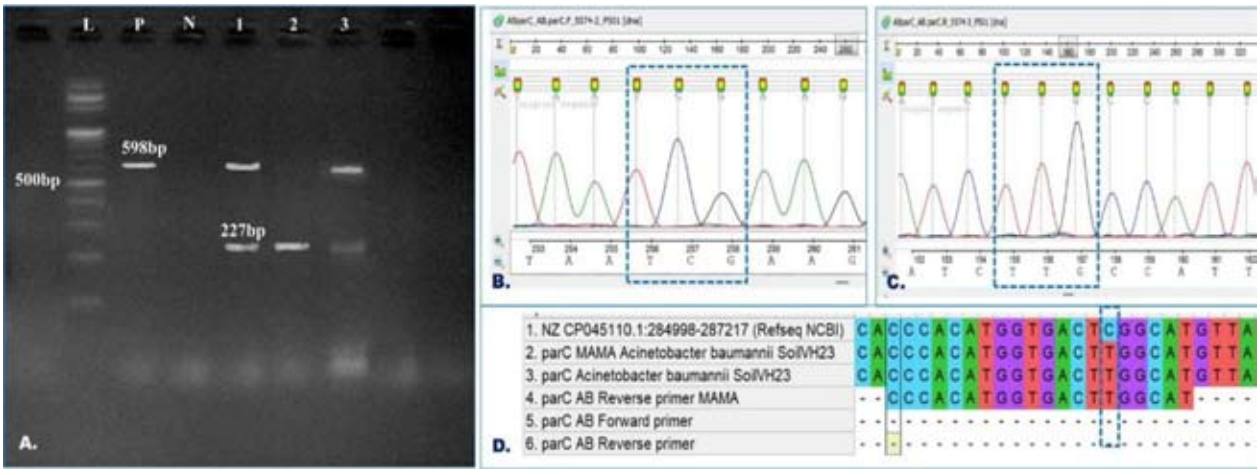

Fig. 5. Validation of *parC* (S84L) MAMA-PCR. (A) Representative agarose gel (2%) of MAMA-PCR results. Lane L: 100 bp DNA ladder; Lane P: Positive control (wild type); Lanes 1-3: Test isolates positive for S84L mutation (598 bp and 227 bp bands- mutants); Lane N: Negative control. (B) Sanger chromatogram of the wild-type isolate, showing the TCG codon. (C) Chromatogram of the mutant isolate, confirming the TTG codon. (D) Multiple sequence alignment showing the single nucleotide polymorphism (MEGA12 software).

However, the study has limitations. The small sample size means that the findings are preliminary. Furthermore, the absence of a mutation-specific amplicon does not rule out the possibility of other mutations within the QRDR or other resistance mechanisms (e.g., efflux pumps) contributing to the observed phenotypic resistance. For definitive confirmation, further validation of these results could be achieved through whole genome sequencing to identify the specific nucleotide substitutions. Additionally, techniques like melt curve analysis could offer an alternative real-time PCR-based confirmation method. Despite these limitations, MAMA-PCR serves as an excellent screening tool to quickly assess the prevalence of known, common resistance mutations in a population of isolates, which is particularly useful for epidemiological tracking.

Conclusion

This study investigated a small group of quinolone-resistant clinical *Acinetobacter baumannii* isolates. The application of a three-primer MAMA-PCR successfully detected the presence of the S81L mutation in the *gyrA* gene and the S84L mutation in the *parC* gene in the majority of resistant isolates. This highlights the utility of MAMA-PCR as a rapid screening method for investigating key resistance mechanisms in *A. baumannii*, which can aid in surveillance and infection control efforts.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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