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Integrated Phenotypic and Molecular Profiling Reveals Strain-Specific Susceptibility to Novel Antimicrobial Agents in Clinical Isolates

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ABSTRACT

The rise of multiple drug resistant (MDR) bacteria requires a shift from conventional diagnostics to integrated methods that can help explain unpredictable strain-specific antimicrobial responses. This study employed a multi-method characterization strategy of clinical isolates to investigate divergent susceptibility to novel agents. Clinical isolates of *Enterococcus faecalis*, *Bacillus cereus*, and two *Proteus mirabilis* strains were obtained from Sacred Heart Hospital, Abeokuta. Comprehensive profiling included biochemical identification, 16S rRNA gene sequencing, phylogenetic reconstruction, and restriction enzyme analysis. Susceptibility to *Nigella sativa* seed oil (NSO), biosynthesized silver nanoparticles (AgNPs), and silver nitrate was evaluated using broth micro-dilution and agar well diffusion assays. Phylogenetic analysis confirmed that the two *P. mirabilis* strains were closely related though restriction enzyme mapping revealed distinct, strain-specific molecular fingerprints. Antimicrobial susceptibility testing showed significant variation. *E. faecalis* was most susceptible to AgNPs, while *P. mirabilis* FELIX004 was completely resistant to AgNPs even though susceptible to both NSO and silver nitrate, a paradoxical profile not seen in the closely related susceptible strain FELIX003. Comparative analysis is suggestive of an association between unique restriction patterns in FELIX004 and its AgNP-resistant phenotype. Integrative phenotypic and molecular profiling uncovered substantial intraspecies variation in novel antimicrobial susceptibility testing. The specific paradoxical resistance of *P. mirabilis* FELIX004 to AgNPs underscores the emergence of agent-specific resistance mechanisms. The study demonstrates that standard phylogenetic relatedness is insufficient to predict strain-specific behavior and highlights the value of multiple approaches for identifying phenotypic outliers that warrant deeper genomic investigation.

Keywords: Clinical isolates; Integrative profiling; 16S rRNA sequencing; Restriction enzyme mapping; Phylogenetic analysis; Antimicrobial susceptibility; Strain-specific resistance; *Proteus mirabilis*; Nanoparticle resistance.

INTRODUCTION

The escalating crisis of antimicrobial resistance (AMR) contributes to around 1.27 million deaths per year, highlighting the critical need for novel approaches to combating multidrug-resistant (MDR) pathogens (Murray et al., 2022). Clinical environments particularly exacerbate this problem as intense antibiotic usage and pathogen transmission pathways, facilitate the development of resistant bacterial strains. An effective counter measure to

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these threats requires diagnostic approaches that move beyond traditional methods to explain unpredictable, strain-specific responses to both conventional and novel antimicrobial agents.

Considerable diversity in virulence and resistance exists among bacterial strains of the same species, stemming from their genetic adaptability facilitated by mechanisms such as horizontal gene transfer and mutational events (Partridge et al., 2018). While essential for classification, conventional methods like 16S rRNA gene sequencing and biochemical profiling can fail to resolve this strain-level diversity, often overlooking the specific genetic determinants that directly influence phenotype (Břinda et al., 2020). Consequently, a more integrative approach which combines phenotypic assays with molecular typing techniques such as restriction analysis and phylogenetic reconstruction becomes necessary for building comprehensive pathogen profiles (Ellington et al., 2017).

This integrated approach is particularly important when evaluating next-generation antibiotics, including plant-derived compounds like *Nigella sativa* oil and biosynthesized nanoparticles, which frequently display unexpected strain-dependent activity (Muteeb et al., 2023). A persistent question is how genetically similar isolates of the same species can demonstrate completely different susceptibility patterns, an indication of a subtle genomic variations, differential gene expression or acquired resistance elements not identified by an extensive phylogenetic investigation (López et al., 2021).

Proteus mirabilis is commonly implicated in catheter-associated urinary tract infections which exemplifies this strain-level complexity. It is associated with coordinated swarming movement, ability to utilize urea and tendencies towards genomic rearrangement. It also displays marked isolate-to-isolate variation in both virulence and antimicrobial susceptibility profiles (Schaffer & Pearson, 2017; Drzewiecka, 2016). The recent discovery of carbapenem-resistant and ESBL-producing strains further affirms its status as a priority pathogen within the AMR ecosystem (Mathers et al., 2023).

This study was therefore designed to determine if an integrative, multi-method characterization strategy could identify and explain divergent antimicrobial susceptibility profiles among clinical isolates, including phylogenetically related strains. We employed a combination of conventional microbiology, molecular biology, and comparative analysis to profile clinical isolates and assess their susceptibility to agents derived from *Nigella sativa* and biosynthesized silver nanoparticles. The central objective was to characterize and compare these isolates in order to identify and elucidate the basis for strain-specific antimicrobial susceptibility patterns that are not predicted by standard identification or phylogenetic relatedness.

MATERIALS AND METHODS

Bacterial Isolation

Clinical bacterial isolates were obtained from the Medical Microbiology and Parasitology Unit of Sacred Heart Hospital in Abeokuta, Ogun State Nigeria, over a six-month surveillance period (January-June 2023). Isolates were collected from blood, cerebrospinal fluid, sputum, urine, and feces following standard aseptic techniques for specimen collection (Lopansri & Bhavsar, 2022). Isolates were initially cultured on appropriate selective media and subjected to standard microbiological identification (Forbes et al., 2022). From an initial collection, eight isolates with distinct morphological characteristics were selected for comprehensive characterization. All selected isolates were maintained in 20% glycerol at -80°C for extended storage following established protocols and were revived on Mueller-Hinton agar (Humphries et al., 2021) prior to experimental use..

Phenotypic Characterization

Comprehensive biochemical profiling (Gram staining, Spore forming, capsule staining, coagulase test, catalase test, indole test, citrate utilization test, motility test, urease test, oxidase test, carbohydrate fermentation test) was performed using standardized protocols (Forbes et al., 2022).

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Antimicrobial Susceptibility Profiling

In line with CLSI guidelines (2018), antimicrobial susceptibility testing was carried out to profile the isolates. Antibiotics tested include ampicillin (10 µg), gentamicin (10 µg), cefotaxime (30 µg), imipenem (10 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg) and vancomycin (30 µg). An isolates was considered MDR if resistant to three or more antibiotic classes.

Genomic Characterization

DNA Extraction and Quality Assessment

Bacterial DNA from overnight cultures was obtained using Wizard Genomic DNA Purification Kit (Promega, USA) in accordance to the manufacturer's instructions (Sambrook & Russell, 2022). DNA quality assessment was by visualization of a single, high-molecular-weight band following electrophoresis on 0.8% agarose gel (Green & Sambrook, 2020).

16S RRNA Gene Amplification and Sequencing

The bacterial 16S rRNA gene amplification was done using PCR with universal primers 27F and 1492R (Weisburg et al., 1991). Thermo-cycling included initial denaturation (95°C, 5 min); 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s with a final extension (72°C, 10 min) (Johnson et al., 2019). PCR products were purified using QIAquick PCR Purification Kit and sequenced bi-directionally using Sanger sequencing (Sanger et al., 1977).

Sequence Analysis and Phylogenetic Reconstruction

Sequence editing was performed in BioEdit v7.2.5. Edited sequences were assessed against the NCBI nucleotide database through BLASTn for preliminary identification based on percent identity and query coverage. A multiple sequence alignment was generated using ClustalW and a phylogenetic tree was constructed in MEGA X using the neighbor-joining method and the Kimura 2-parameter model. Branch support was assessed with 1000 bootstrap replicates (Kumar et al., 2018).

Restriction Enzyme Analysis

NEBcutter version 3.0 was used to carry out In silico restriction enzyme mapping with complete 16S rRNA gene sequences as input (Vincze et al., 2003). PCR-amplified 16S rRNA genes were digested with EcoRI, HindIII, and BamHI (New England Biolabs, USA). 2% agarose gel electrophoresis was used to separate digestion products before visualization with ethidium bromide staining under UV light.

Molecular Weight Determination

DNA and RNA molecular weights were calculated using established formulas based on nucleotide composition. For double-stranded DNA: MW = (number of A nucleotides × 313.2) + (number of T nucleotides × 304.2) + (number of G nucleotides × 329.2) + (number of C nucleotides × 289.2). For single-stranded RNA: MW = (number of A nucleotides × 329.2) + (number of U nucleotides × 306.2) + (number of G nucleotides × 345.2) + (number of C nucleotides × 305.2). Nucleotide frequencies were determined from 16S rRNA sequences (Cantor & Schimmel, 1980).

Antimicrobial Susceptibility Testing to Novel Agents

All antimicrobial agents were prepared according to standardized protocols to ensure consistency and reproducibility (Balouiri et al., 2021). *Nigella sativa* seed oil (NSO) was extracted from dried seeds using a Soxhlet apparatus with n-hexane as the solvent, following an established protocol for obtaining bioactive oil (El-Maati et al., 2012). Biosynthesized silver nanoparticles (AgNPs) were produced using a green synthesis method. A 10% (v/v) NSO solution was reacted with a 5 mM aqueous silver nitrate ($AgNO_3$) solution under ambient sunlight exposure, facilitating the phytochemical-mediated reduction of silver ions (Khan et al., 2021). For

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comparative analysis, a standard 5 mM aqueous solution of $AgNO_3$ was prepared to serve as an ionic silver control (Slavin et al., 2022). A 5 µg/mL aqueous solution of ciprofloxacin was prepared as a positive control in all antimicrobial assays to validate assay performance (CLSI, 2023).

Agar Well Diffusion Assay

Bacterial suspensions were adjusted to a 0.5 McFarland standard. Mueller-Hinton agar plates were swab-inoculated and 6 mm wells were aseptically punched using sterile cork borer. Aliquots (100 µL) of each test agent at 100%, 75%, 50%, and 25% concentrations were dispensed into the corresponding wells using a micropipette. Following incubation at 37°C for 24 hours, the zones of inhibition (ZOI) were measured in millimeters in accordance with CLSI guidelines (CLSI, 2023).

Minimum Inhibitory and Bactericidal Concentrations (MIC/MBC) Assessment

MIC assay: The minimum inhibitory concentration (MIC) was determined using broth microdilution method in 96-well plates following CLSI guidelines (CLSI, 2023). Two-fold serial dilutions of each antimicrobial agent (100% to 0.05%) were prepared and along with previously standardized bacterial suspension (5×10^5 CFU/mL) dispensed using a micropipette and incubated at 37°C for 24 hours. The MIC was recorded as the lowest concentration preventing visible growth.

MBC assay: The minimum bactericidal concentration (MBC) was subsequently determined by subculturing from each clear well of the MIC study onto Mueller-Hinton agar. The MBC was determined to be the lowest concentration that achieved a ≥99.9% reduction in the initial viable inoculum after 24 hour incubation at 37°C.

Statistical and Bioinformatic Assessment of Results

Statistical analysis was carried out on the result using GraphPad Prism version 9.0. Variations in responses among strains for each antimicrobial agent were analyzed utilizing One-way Analysis of Variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) and then Post hoc test for Multiple Comparisons (McDonald, 2023). The correlation between genomic features and susceptibility metrics was assessed using the Pearson correlation coefficient (McDonald, 2023). Bioinformatic analysis carried out included multiple sequence alignment, phylogenetic reconstruction, restriction site prediction and comparative genomics using established algorithms within the MEGA X and BioEdit software suites (Kumar et al., 2021) and following standard bioinformatics principles (Mount, 2023).

RESULT

Phenotypic Characterization of Clinical (Bacteria) Isolates

Comprehensive biochemical profiling identified four distinct bacterial isolates from the initial eight candidates as depicted in Table 1

Table 1: Biochemical Characterization of Clinical Isolates

ID	GR	SP	CA	CT	CO	MO	IN	OX	CI	UR	H_2S	MR	VP	G	L	S	M	Organism
A1	GNB	–	–	+	–	+	–	–	+	+	–	+	+	A	A	A	A	Klebsiella sp.
A2	GNB	–	–	–	–	+	+	–	–	–	–	+	–	A	A	–	A	Escherichia sp.
A3	GPB	+	+	+	–	+	–	–	+	–	–	+	–	A	–	–	–	Bacillus sp.
A4	GNB	–	–	+	–	–	–	–	+	–	–	–	+	A	A	–	–	Pseudomonas sp.

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Keys: A, Acid production; CA, Capsule staining; CI, Citrate utilization; CT, Catalase test; G, Glucose; GR, Gram staining; H_2S, Hydrogen sulfide production; IN, Indole test; L, Lactose; M, Mannitol; MO, Motility test; MR, Methyl red test; OX, Oxidase test; S, Sucrose; SP, Spore staining; UR, Urease test; VP, Voges-Proskauer test.

Symbols: –, Negative; +, Positive.

Each of the four isolates exhibited unique phenotypic characteristics which led to subsequent molecular analysis to confirm their preliminary biochemical classifications. Isolate A1 exhibited a classic profile for *Enterobacteriaceae*: a motile, Gram-negative bacillus that was urease, citrate, and hydrogen sulfide positive while fermenting both glucose and lactose, traits that are suggestive of *Klebsiella* sp., but subsequent molecular analysis necessitated reclassification. In contrast, Isolate A2 presented a divergent profile within the same family. This motile, Gram-negative bacillus was indole and methyl red positive but negative for citrate, urease, and hydrogen sulfide production and it fermented only glucose. This pattern initially indicated *Escherichia* sp., though molecular methods revealed a different identity. Isolate A3 was clearly differentiated as a Gram-positive organism. Its morphology as a spore-forming bacillus, coupled with positive results for catalase, oxidase, motility, citrate, urease, and hydrogen sulfide, was consistent with the genus *Bacillus*, a finding later confirmed molecularly. Finally, Isolate A4 presented a more complex biochemical picture. This non-motile, Gram-negative bacillus was catalase and oxidase positive, fermented both glucose and lactose, and produced hydrogen sulfide, leading to an initial identification as *Pseudomonas* sp. However, its sugar fermentation capability was atypical for this genus, a discrepancy resolved by molecular reclassification.

Molecular Characterization and Phylogenetic Analysis

16S rRNA Gene Sequencing and BLAST Analysis providing definitive species identification as depicted in table 2 below;

Table 2**:** Molecular Characterization of the Clinical (Bacteria) Isolates

Name of samples	Percentage ID (%)	Accession No	Strain No	Bacteria
A1	96.29	NR-114419.1	FELIX003	Proteus mirabilis
A2	96.96	NR-115765.1	FELIX001	Enterococcus feacalis
A3	94.49	NR-157734.1	FELIX002	Bacillus cereus
A4	98.76	NR-114419.1	FELIX004	Proteus mirabilis

The discrepancy between biochemical and molecular identification for isolates A1 and A4 highlights limitations of conventional phenotypic methods, particularly for closely related Enterobacteriaceae members.

Phylogenetic Reconstruction

Evolutionary connections between the isolates were elucidated through phylogenetic reconstruction using their 16S rRNA gene sequences as depicted in figure 1 below;

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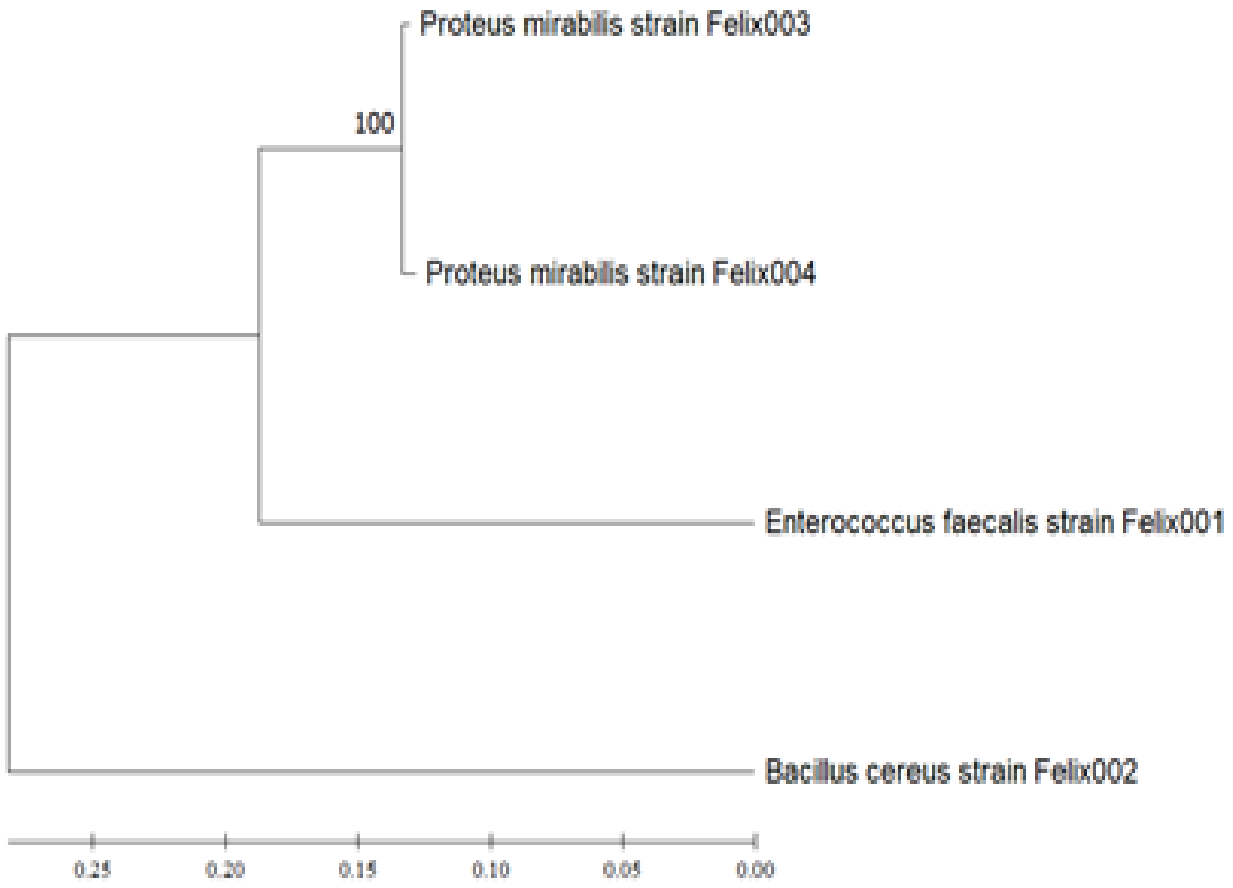

Figure 1: Phylogenetic tree of identified bacteria strains

The phylogenetic tree showed clearly the evolutionary relationships among the isolates. The two *P. mirabilis* strains, FELIX003 and FELIX004, formed a tight, well-supported clade with 100% bootstrap value, confirming a close evolutionary relationship and high genetic similarity at this locus. *E. faecalis* FELIX001 occupied a distinct branch, phylogenetically separated from the Gram-negative cluster. As expected, *B. cereus* FELIX002 demonstrated the greatest evolutionary distance from all other isolates, consistent with its taxonomic classification in a different genus and Gram-positive lineage.

Significantly, this clear phylogenetic clustering stood in direct contrast to the observed phenotypic data. Despite their close evolutionary relationship and genetic similarity, strains FELIX003 and FELIX004 exhibited marked differences in their susceptibility to the tested antimicrobial agents. This paradoxical finding—where phylogenetically similar strains displayed divergent phenotypes—highlighted the limitations of 16S rRNA analysis alone for predicting antimicrobial susceptibility and necessitated deeper genomic investigation to uncover the basis for this differential response

Genomic Characterization

Restriction Enzyme Mapping

In silico restriction analysis of 16S rRNA gene sequences revealed distinct patterns as depicted in fig. 2 below

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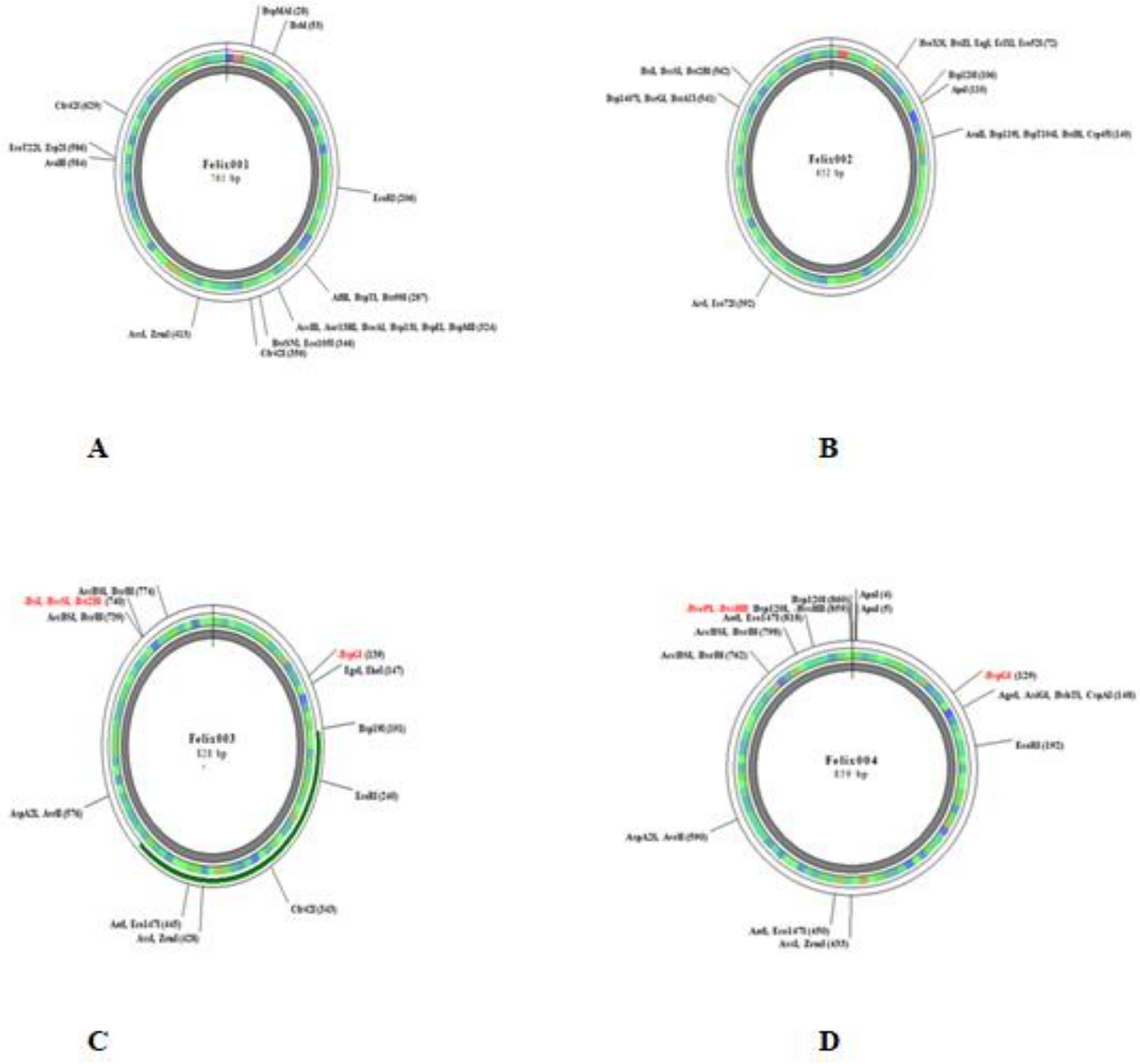

Fig. 2: Showing the restriction enzymes for A- *Enterococcus faecalis* (FELIX001), B- *Bacillus cereus* (FELIX002), C- *Proteus mirabilis* (FELIX003) and D- *Proteus mirabilis* (FELIX004)

The restriction enzyme analysis of the 16S rRNA gene region showed distinct genomic fingerprints for each isolate, uncovering diversity that was not apparent from sequence similarity alone.

Enterococcus faecalis FELIX001 (761 bp) displayed a unique profile characterized by multiple EcoRI restriction sites, distinguishing it from the other isolates. In contrast, *Bacillus cereus* FELIX002 (652 bp) was uniquely defined by the presence of BsrBI sites.

Notably, the two *Proteus mirabilis* strains, which were closely related by 16S rRNA phylogeny, showed markedly different restriction patterns. FELIX003 (828 bp) displayed a relatively simpler profile, with multiple BssHII sites indicating GC-rich regions. Conversely, FELIX004 (859 bp) presented the most complex and unique fingerprint, containing several distinctive sites, including DrdII, Bsp191, AseI, and AsuNHI that were absent in its counterpart. This stark contrast in restriction profiles between the two *P. mirabilis* strains revealed a level of underlying genomic diversity that was not captured by their high 16S rRNA sequence similarity.

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Molecular Weight Analysis

Table 3: Deoxyribonucleic acid (DNA) Molecular Weight of the Isolated Bacteria

Organisms	Molecular Weight (g/mol)	Total bases	DNA Ratio (A:G:T:C)
Enterococcus faecalis Felix001	460,975	1522	23:27:23:27
Bacillus cereus Felix002	395,043	1304	23:27:23:27
Proteus mirabilis Felix003	501,774	1656	24:26:24:26
Proteus mirabilis Felix004	520,491	1718	23:27:23:27

Key: A:G:T:C means Adenine: Guanine: Thymine: Cytosine

Table 4: Ribonucleic acid (RNA) Molecular Weight of the Isolated Bacteria

Organisms	Molecular Weight (g/mol)	Total bases	DNA Ratio (A:G:T:C)
Enterococcus faecalis Felix001	228,256	761	20:22:26:32
Bacillus cereus Felix002	198,120	652	26:28:21:25
Proteus mirabilis Felix003	248,682	828	22:22:26:31
Proteus mirabilis Felix004	257,577	859	21:21:26:32

Key: A:G:U:C means Adenine: Guanine: Uracil: Cytosine

Molecular weight analysis of the genomic DNA and 16S rRNA amplicons as depicted in tables 3 and 4 above revealed distinct quantitative differences among the isolates. For the DNA molecules, *E. faecalis* FELIX001 had the largest molecular weight at 460,975 g/mol (1522 bases), while *B. cereus* FELIX002 possessed the smallest at 395,043 g/mol (1304 bases), despite both sharing an identical nucleotide composition (A:G:T:C ratio of 23:27:23:27). The two *P. mirabilis* strains showed a clear stepwise increase in size; FELIX003 weighed 501,774 g/mol (1656 bases) with a slightly different base ratio of 24:26:24:26, and FELIX004 was the largest at 520,491 g/mol (1718 bases), reverting to the 23:27:23:27 ratio. This pattern was mirrored in the RNA data, where *E. faecalis* also contained the largest rRNA fragment (228,256 g/mol; 761 bases) and *B. cereus* the smallest (198,120 g/mol; 652 bases). While *P. mirabilis* FELIX004 again possessed a larger rRNA molecule (257,577 g/mol) than FELIX003 (248,682 g/mol), both shared a notably higher cytosine and uracil content compared to the other isolates, as indicated by their base ratios. Overall, the findings demonstrate significant variation in the molecular size of conserved genetic markers between species and, critically, between the closely related *P. mirabilis* strains.

Antimicrobial Susceptibility to Novel Agents

Table 5: Antibacterial Activity of the Extracts against the organisms

Organism	Extract Concentrations (%) Mean Zone Diameter (mm)												
	Nigella sativa				Biosynthesized silver nanoparticles				Silver nitrate solution				Control
	100	75	50	25	100	75	50	25	100	75	50	25	
Enterococcus faecalis	17.33± 0.8^{a}	13.00± 0.6^{a}	9.33 ± 0.3^{a}	0.00 ± 0.0^{a}	23.00± 0.5^{c}	14.67± 0.6^{b}	13.67± 0.6^{b}	11.00± 0.5^{c}	21.00± 0.5^{b}	17.33± 0.8^{b}	14.00± 0.5^{c}	12.00± 0.5^{b}	29.33 ± 0.3^{a}
Bacillus cereus	21.00± 0.5^{bc}	17.33± 0.3^{b}	11.67± 0.6^{b}	0.00 ± 0.0^{a}	19.00± 0.5^{b}	15.33± 0.8^{b}	12.33± 0.8^{b}	9.00 ± 0.5^{b}	16.00± 0.5^{a}	12.00± 0.5^{a}	9.00 ± 0.5^{a}	0.00 ± 0.0^{a}	34.00 ± 1.1^{b}

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Proteus mirabilis	22.33± 0.8^{c}	20.00± 0.5^{c}	16.00± 0.5^{c}	12.00± 0.5^{b}	18.00± 0.5^{b}	15.33± 0.8^{b}	11.33± 0.8^{b}	9.33 ± 0.3^{b}	16.67± 0.3^{a}	15.33± 0.8^{b}	11.67± 0.3^{b}	11.00± 0.5^{b}	29.67 ± 0.3^{a}
Proteus mirabilis	19.00± 0.5^{ab}	16.67± 1.2^{b}	11.00± 0.5^{ab}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	24.33± 0.6^{c}	21.33± 0.3^{c}	17.67± 0.3^{d}	14.33± 0.8^{c}	27.67 ± 0.8^{a}

Key: Values represent the mean ± SEM (n=3)

Within a column, mean values followed by different superscript letters are significantly different ($p < 0.05$)The antimicrobial susceptibility testing (Table 5) revealed significant and unexpected strain-specific variation among the isolates. *Enterococcus faecalis* FELIX001 demonstrated the greatest susceptibility to the biosynthesized silver nanoparticles (AgNPs) with an MIC of 12.5µg/mL and showing moderate susceptibility to the other agents. In contrast, *Bacillus cereus* FELIX002 and *Proteus mirabilis* FELIX003 were most susceptible to *Nigella sativa* oil (NSO).

The most critical finding involved the two phylogenetically related *P. mirabilis* strains. While FELIX003 showed a moderate susceptibility pattern, FELIX004 exhibited a paradoxical resistance profile. It was completely resistant to AgNPs at all tested concentrations yet remained susceptible to precursor materials, NSO and silver nitrate ($AgNO_3$). This AgNP-specific resistance in a strain susceptible to the individual components represents a key finding requiring genomic investigation.

A detailed analysis of the dose-response curves further highlighted unique strain behaviors. While all isolates showed reduced activity at lower concentrations, the rate of this decline varied. For instance, *P. mirabilis* FELIX003 maintained some antimicrobial activity even at a 25% concentration for all agents, a persistence not observed in the other isolates. Furthermore, the primary mode of action differed for one critical combination; for AgNPs against *P. mirabilis* FELIX003, the high MBC/MIC ratio suggested a bacteriostatic effect, whereas most other agent-strain combinations demonstrated bactericidal activity.

Genomic-Phenotypic Correlations

A comparative analysis of genomic and phenotypic data revealed several potential correlations between specific genetic markers and the observed antimicrobial susceptibility profiles.

A notable finding was the association between unique restriction enzyme sites and resistance to silver nanoparticles (AgNPs). *Proteus mirabilis* FELIX004, which exhibited complete resistance to AgNPs, possessed a distinct set of restriction sites—including DrdII, Bsp191, AseI, and AsuNHI—that were absent in all susceptible strains. This suggests these specific genomic regions may harbor determinants for nanoparticle-specific resistance.

Furthermore, an analysis of GC content indicated a potential trend with susceptibility. The two *P. mirabilis* strains possessed a higher GC content (48%) compared to *E. faecalis* and *B. cereus* (both 44%). This higher genomic GC content correlated with an overall lower susceptibility to AgNPs across the isolates. Conversely, there was no noticeable relationship found between the molecular weight of the nucleic acid amplicons and antimicrobial susceptibility. Critically, the analysis confirmed that antimicrobial response was not a phylogenetically conserved trait. Despite forming a tight evolutionary clade, the two *P. mirabilis* strains (FELIX003 and FELIX004) displayed markedly divergent susceptibility patterns, particularly to AgNPs. This underscores that closely related strains can evolve or acquire distinct resistance mechanisms that are not predicted by broad phylogenetic relationships.

DISCUSSION

The inconsistency observed between preliminary biochemical identification and subsequent molecular validation accentuate recognized constraints of traditional phenotypic approaches, especially when assessing

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closely related members of the Enterobacteriaceae family like *Proteus mirabilis* (Janda & Abbott, 2002; 2022). *P. mirabilis* is notoriously variable in key characteristics like swarming motility and carbohydrate fermentation, which can lead to misidentification (O'Hara et al., 2000). Our findings reinforce the need for molecular verification for accurate species identification in a research context.

Consistent with recent observations (Kvitek et al., 2020; Hirose et al., 2022), restriction enzyme mapping can provide a valuable layer of differentiation not visible from sequence analysis alone. While the two P. mirabilis strains appear closely related, upon examination of their gene sequences and position on the phylogenetic tree, their restriction profiles were clearly distinct. The unique combination of sites (e.g., DrdII, Bsp191) in FELIX004 suggests underlying sequence divergence or structural differences in this conserved region, potentially indicative of strain-level genomic rearrangements as recently documented in Proteus mirabilis (Cruz-Córdova et al., 2022).This demonstrates that restriction analysis while less comprehensive than whole-genome sequencing, can serve as a rapid and effective tool for strain differentiation and for generating hypotheses about genomic diversity (Olive & Bean, 1999).

A key discovery from this investigation was the absolute and selective resistance displayed by P. mirabilis FELIX004 toward biosynthesized silver nanoparticles (AgNPs), even though this strain remained susceptible to both precursor compounds, Nigella sativa oil (NSO) and silver nitrate solution. This self-contradictory phenotype, also documented by El-Batal et al. (2020) in P. mirabilis exposed to Nigella sativa-synthesized AgNPs, suggests a resistance mechanism specific to the nanoparticle form or its phytochemical capping. Several non-exclusive mechanisms could explain this observation, which is consistent with known bacterial adaptive strategies (El-Batal et al., 2020; Panáček et al., 2018). Panáček et al. (2018) further demonstrated that Proteus strains exhibit nanoparticle-specific resistance while remaining susceptible to ionic silver, mirroring the phenotype observed in FELIX004. The modified surface of AgNPs, coated with NSO phytochemicals, may be recognized differently by bacterial cells. FELIX004 could possess altered surface structures like lipopolysaccharide or outer membrane proteins that reduce nanoparticle adhesion or uptake (Wang et al., 2017). Furthermore, the enhancement of efflux pumps capable of exporting the coated nanoparticles is a probable resistance strategy that may be commonly employed by Enterobacteriaceae (Du et al., 2018). Alternatively, enhanced antioxidant defenses or biofilm production in FELIX004 could mitigate the oxidative stress or physical penetration typically induced by AgNPs (Dwyer et al., 2014; Jones et al., 2004). The distinct restriction profile of FELIX004 provides a molecular marker that fits this unusual phenotype, flagging it as a strain requiring further investigation to pinpoint the exact genetic basis of its resistance.

The observation of divergent susceptibility between phylogenetically similar P. mirabilis strains has direct implications. It hints at a critical limitation of relying on species-level identification or broad phylogenetic markers for predicting responses to novel antimicrobial agents such as those of nanoscale origin (Bankier et al., 2021; Arya & Mishra, 2023). Strain-specific responses as demonstrated here could prove problematic for the development and deployment of such agents if not accounted for during evaluation (Panáček et al., 2018; Qais et al., 2021).

Finally, this study alludes to the practical utilization of an integrative, multi-method profiling strategy. By combining conventional biochemistry, molecular phylogeny and restriction typing, we were able to correctly identify isolates, establish their evolutionary relatedness, uncover hidden strain-level diversity and identify a strain (FELIX004) with a clinically and mechanistically interesting resistance profile. This framework is efficient for preliminary characterization and for prioritizing strains for more resource-intensive genomic analyses (Gao et al., 2023).

CONCLUSION

The study applied an integrative phenotypic and molecular profiling strategy to clinical bacterial isolates thus revealing significant and unpredictable strain-specific variation in susceptibility to novel antimicrobial agents. The central discovery was the paradoxical resistance profile of *Proteus mirabilis* FELIX004, which remained susceptible to both *Nigella sativa* oil and ionic silver but exhibited complete resistance to biosynthesized silver nanoparticles. The small number of isolates utilized prevents this generalization even though study finding

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highlights the emergence of agent-specific resistance mechanisms that could be missed by conventional antimicrobial testing practice.

The research clearly demonstrates that close phylogenetic relatedness as determined by 16S rRNA sequencing, was insufficient to predict these strain-specific phenotypic outcomes. The divergent susceptibility between the closely related *P. mirabilis* FELIX003 and FELIX004 stresses the critical importance of moving beyond species-level identification in both research and diagnostic contexts. Whole genome sequencing would have provided a more definitive outcome, pin-pointing the genetic determinants responsible for the observed resistance pattern and their mechanism of action.

The multi-method framework employed synthesizing biochemical tests, molecular typing and comparative analysis helped prove the effectiveness of comprehensive pathogen profiling. It was particularly efficient at identifying phenotypic outliers and correlating them with distinct molecular fingerprints such as unique restriction enzyme patterns. This approach provides a model for initial cost-effective characterization that can pin-point high-priority strains for subsequent thorough genomic investigation to elucidate the precise mechanisms underlying unique resistance phenotypes like that observed in *P. mirabilis* FELIX004.

Conflict of Interest: The authors declare no conflict of interest.

Authors Contribution:

1. Olanlege, A.O. was involved in study design, statistical analysis, data interpretation, manuscript preparation, literature search

2. Olaoye, F.A. was involved in study design, data collection, statistical analysis, data interpretation, manuscript preparation, literature search and fund collection

3. Sanusi, J.F. was involved in study design and data interpretation

4. Sirajudeen, A.O. was involved in Manuscript preparation

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Data Availability Statement: The 16S rRNA sequences generated in this study have been deposited in GenBank. All other data are available upon reasonable request from the corresponding author.

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