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Molecular Identification and Phylogenetic Analysis of Fluoroquinolone-Resistant Bacteria Isolated from Poultry Manure-Amended Agricultural Soils in Khorasan Razavi Province, Iran

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ABSTRACT

Long-term poultry manure application introduces strong selective pressure and persistent veterinary antibiotic residues into agricultural soils, establishing critical environmental antimicrobial resistance hotspots. This study investigated the molecular taxonomy, phenotypic traits, and phylogenetic diversity of fluoroquinolone-resistant bacteria isolated from manure-amended fields in Khorasan Razavi Province, northeastern Iran. Cultivation on ciprofloxacin-supplemented selective media yielded three resistant bacterial isolates; among these, a distinct Gram-negative coccobacillus exhibited high-level phenotypic resistance with an exceptionally high minimum inhibitory concentration (MIC) of 35 µg/mL. Bidirectional Sanger sequencing successfully recovered a 1,411 bp fragment of its 16S rRNA gene. Although preliminary BLASTn alignment analysis showed a high sequence identity of 98.87% to *Acinetobacter baumannii* reference strains, a global Maximum Likelihood phylogenetic tree reconstructed using RAxML with 500 bootstrap replicates definitively revealed that the isolate does not cluster within the strict A. baumannii monophyletic clade (97% support). Instead, it occupies a unique, transitional phylogenetic position as a sister lineage to *Acinetobacter soli* and the core *A. baumannii* complex, leading to its taxonomic classification as *Acinetobacter* sp. strain NEY04 (GenBank: PZ542786). Ultimately, given the prominent role of this genus in driving horizontal gene transfer, these findings establish a vital evolutionary baseline for future down-stream sequencing of specific genetic mechanisms within environmental agro-ecosystems.

Introduction

Antimicrobial resistance (AMR) represents one of the most pressing global public health challenges of the 21st century (Murray et al., 2022). The rapid emergence and dissemination of antibiotic-resistant bacteria have been driven by the overuse and misuse of antimicrobial agents in human medicine, animal husbandry, and agriculture (Ghimpețeanu et al., 2022). Within the One Health framework, the environment particularly agricultural soils, serves as a critical reservoir and hotspot for the evolution and horizontal transfer of resistance determinants (Nesme & Simonet, 2015).

Fluoroquinolones are broad-spectrum synthetic antibiotics widely used in both human and veterinary medicine (Neu, 1992). In poultry production, enrofloxacin and other fluoroquinolones are frequently administered for

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the prevention and treatment of respiratory and enteric infections (Vargas et al., 2026). A significant portion of these antibiotics is excreted in active form through feces, leading to the accumulation of antibiotic residues in poultry manure. When this manure is applied as an organic fertilizer to agricultural fields, it introduces both selective pressure and resistant bacteria into the soil environment which can significantly enrich fluoroquinolone-resistant populations (Wang et al., 2024).

In Iran, poultry farming is a major component of the agricultural sector (Shariatmadari, 2000). Khorasan Razavi province, located in northeastern Iran, is characterized by intensive poultry production and extensive use of poultry manure as a low-cost fertilizer in surrounding farmlands similar to other regions of Iran (Mohammadi et al., 2025). Many agricultural fields in this region are located in close proximity to commercial poultry farms and have received regular applications of untreated or partially composted poultry litter for several years. Such agro-ecosystems create favorable conditions for the persistence and proliferation of antibiotic-resistant bacteria, including clinically important opportunistic pathogens.

Opportunistic bacteria capable of thriving in poultry manure and manure-amended soils are frequently exposed to antibiotic residues, which exerts strong selective pressure favoring the development and maintenance of resistance. Many of these environmental isolates possess high genomic plasticity, enabling them to readily acquire and disseminate antimicrobial resistance determinants through horizontal gene transfer (Palmer et al., 2010). This positions manure-impacted agricultural soils as potential hotspots for the evolution and spread of resistance traits from environmental reservoirs to clinically relevant settings. Although numerous studies have examined antibiotic resistance patterns among clinical isolates in Iran (Jabbari et al., 2025), relatively little attention has been given to the molecular taxonomy and phylogenetic diversity of fluoroquinolone-resistant bacteria in poultry manure-amended agricultural soils of this region.

Understanding the taxonomic composition and evolutionary relationships of resistant bacterial populations is essential for assessing their ecological distribution, potential pathogenicity, and public health risk (Woese, 1987). The 16S rRNA gene sequencing approach remains a gold standard for bacterial identification and phylogenetic analysis, providing reliable taxonomic placement and insights into the diversity of resistant isolates. Such baseline information is particularly important in high-risk environments such as manure-amended soils, where it can reveal dominant resistant taxa and guide subsequent mechanistic studies (Janda & Abbott, 2007).

To date, no comprehensive study has characterized the molecular diversity and phylogenetic relationships of fluoroquinolone-resistant bacteria in poultry manure-amended agricultural soils of Khorasan Razavi province. Therefore, the present study aimed to isolate ciprofloxacin-resistant bacteria from these soils, identify them at the molecular level through 16S rRNA gene sequencing, and determine their phylogenetic relationships. This work provides the first taxonomic snapshot of fluoroquinolone-resistant bacteria in these agro-ecosystems and establishes a foundation for further investigations into the genetic mechanisms of resistance in dominant or high-risk isolates.

Methods

Soil Sampling

Soil samples were collected from agricultural fields in Mashhad, Neyshabur, and Sabzevar (Khorasan Razavi Province, northeastern Iran) that had been regularly amended with poultry manure for several years. These farmlands were located near commercial poultry farms. To maintain confidentiality, the specific names of the source farms are not disclosed. Soil samples were collected from the upper layer (0–15 cm) using sterile equipment, placed into sterile plastic bags, and transported to the laboratory on ice. The samples were either processed immediately upon arrival or stored at 4°C until subsequent analysis.

Isolation of Fluoroquinolone-Resistant Bacteria

For the selective isolation of fluoroquinolone-resistant bacteria, soil suspensions were serially diluted in sterile phosphate-buffered saline and inoculated onto Mueller-Hinton agar plates. To ensure selective pressure, the media was supplemented with a fixed concentration of ciprofloxacin at 4 µg/mL and 100 µg/mL of

cycloheximide to inhibit fungal overgrowth (Wakisaka & Koizumi, 1982). To prevent photodegradation of the antibiotic, all plate preparation and subsequent incubations were performed in complete darkness. The plates were incubated aerobically at 28°C for 72 hours to optimize the growth of environmental bacteria. Colonies displaying distinct morphological characteristics (color, size, shape, and margin) were selected and repeatedly streaked onto fresh selective media to obtain pure cultures. Purified isolates were then preserved in 20% glycerol at –80°C for further analysis.

Genomic DNA Extraction

Genomic DNA was extracted from the pure bacterial cultures using the AccuPrep Genomic DNA Extraction Kit (Bioneer Corporation, South Korea) following the manufacturer's protocol. To ensure efficient lysis of potential Gram-positive soil isolates, bacterial pellets were pre-incubated with lysozyme before detergent-mediated cell lysis. The concentration and chemical purity of the eluted genomic DNA were quantified using a NanoDrop spectrophotometer, confirming that the A_{260}/A_{280} ratio fell within the acceptable range of 1.8 to 2.0. DNA structural integrity was visually verified via agarose gel electrophoresis.

PCR Amplification of 16S rRNA Gene

The nearly full-length 16S rRNA gene (~1500 bp) was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and a modifed version of 1492R (5′-GG**Y**TACCTTGTTACGACTT-3′) (Weisburg et al., 1991). PCR amplifications were performed in a total volume of 25 µL. The thermocycling profile consisted of an initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s; with a final extension step at 72°C for 7 min. The resulting PCR products were resolved via electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under a UV transilluminator to confirm the presence of a single band of the expected size.

Sanger Sequencing

Amplicons of the expected size were purified and subjected to bidirectional Sanger sequencing using the flanking primers 27F and 1492R to obtain full-length sequence coverage. Sequencing was performed on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems) by Macrogen LTD. The resulting forward and reverse raw chromatograms were visually inspected for quality, trimmed, and assembled into high-quality consensus sequences using CLC Genomics Workbench 26 software (QIAGEN, USA).

Taxonomic Identification and Phylogenetic Analysis

For preliminary taxonomic screening, the final consensus 16S rRNA gene sequences were queried against reference type strains in the NCBI GenBank and EZBioCloud databases using the BLASTn algorithm. While a sequence similarity threshold of >98.7% was initially targeted for species-level assignment (Kim et al., 2014), comprehensive phylogenetic tree reconstruction was employed to definitively resolve species-level boundaries due to the hyper-conserved nature of the 16S rRNA marker among closely related members of the genus *Acinetobacter*. To determine the exact evolutionary relationships of the isolated strains, a phylogenetic tree was constructed within Geneious Prime (Dotmatics). The 16S rRNA gene sequences from this study were aligned with verified reference and type strain sequences retrieved from GenBank using the MAFFT alignment plugin (Katoh & Standley, 2013). A Maximum Likelihood phylogenetic tree was subsequently reconstructed using the RAxML plugin, employing the GTR GAMMA nucleotide substitution model (Stamatakis, 2014). Topological branch support was evaluated by rapid bootstrap analysis with 500 replicates. The final tree was rooted using a multi-species outgroup cluster composed of *Escherichia coli* K12, *Pseudomonas aeruginosa* ATCC 27853, and *P. aeruginosa* PAO1, and visually optimized within the Geneious Tree Viewer.

Results

Isolation and Morphological Characterization of Fluoroquinolone-Resistant species

A total of three bacterial isolates capable of growing on Mueller-Hinton agar supplemented with ciprofloxacin were obtained from poultry manure-amended agricultural soils in Khorasan Razavi province. Among them, one isolate (designated as Isolate 2) exhibited robust growth on selective media containing 4 µg/mL ciprofloxacin, forming well-isolated colonies within 48 hours of incubation (Fig 1a). This isolate

a

b

Fig 1. (a) Robust colony growth of Acinetobacter sp. strain NEY04 (initially designated as Isolate 2) on selective Mueller-Hinton agar supplemented with 4 µg/mL ciprofloxacin to maintain selective pressure and 100 µg/mL cycloheximide to prevent fungal overgrowth. Plate preparation and the subsequent 48-hour aerobic incubation at 28°C were conducted in complete darkness to avoid antibiotic photodegradation. Visible colonies are small-to-intermediate (1.5–2.5 mm), circular, slightly convex, smooth, opaque, and creamy-white with entire margins and a butyrous texture. (b) Light microscopy view (1000× magnification) of the Gram stain presentation for Isolate 2, revealing distinctive Gram-negative coccobacilli arranged singly, in pairs (diplococci), or forming small clusters, without endospores or filamentous tracking.

displayed a high-level resistance phenotype with a minimum inhibitory concentration (MIC) of 35 µg/mL. In contrast, the other two isolates showed weaker growth on the same selective medium and exhibited only low-level resistance (MIC ≈ 1 µg/mL).

Macroscopic examination of Isolate 2 revealed small to intermediate-sized (1.5–2.5 mm), circular, slightly convex, smooth, opaque, creamy-white to pale ivory colonies with entire margins and a butyrous texture (Fig 1a). Gram staining showed Gram-negative coccobacilli occurring singly, in pairs (diplococci), or small clusters, with no endospores or filamentous structures observed (Fig 1b). These morphological features are characteristic of the genus *Acinetobacter* (Doughari et al., 2011). The isolate was clearly distinguishable from co-existing soil flora such as filamentous actinomycetes on the primary isolation plates.

PCR Amplification and Sanger Sequencing of the 16S rRNA Gene

Genomic DNA was successfully extracted from all three isolates. PCR amplification using universal primers 27F and 1492R yielded a single, sharp band of approximately 1500 bp for each isolate, consistent with the expected size of the nearly full-length 16S rRNA gene (Fig 2).

Bidirectional Sanger sequencing was performed on the amplicon from Isolate 2 (the high-resistance strain). High-quality sequences were obtained with 918 bp from the forward primer and 826 bp from the reverse primer (Fig 3). The assembled consensus sequence reached 1411 bp with excellent quality and no significant noise or ambiguous bases.

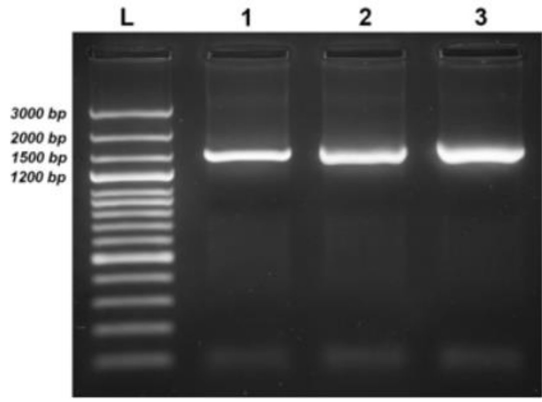

Fig 2. Agarose gel electrophoresis of 16S rRNA gene PCR products (~1500 bp). Lane L: DNA ladder; Lane 1 and 3: low-resistance isolates; Lane 2: high-resistance A. baumannii Isolate 2.

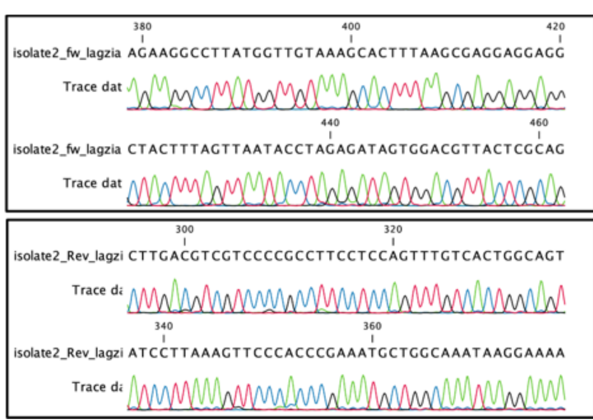

Fig 3. Bidirectional Sanger sequencing raw chromatogram profiles Representative high-resolution fluorogram sequencing traces capturing portions of the forward read (top panel, utilizing primer 27F yielding 918 bp) and reverse read (bottom panel, utilizing primer 1492R yielding 826 bp) generated from the Isolate 2 PCR product using an ABI Prism 3730xl Genetic Analyzer. The clean, distinct peak resolutions validate the exceptional quality metrics, lack of background noise, and absence of ambiguous bases across the sequencing run. These bidirectional reads were edited, trimmed, and assembled into the final 1,411 bp high-quality consensus sequence within the CLC Genomics Workbench 26 suite prior to downstream database alignment queries.

Taxonomic Identification of the Isolates

Preliminary BLASTn analysis of the 1411 bp 16S rRNA gene sequence from Isolate 2 showed 100% query coverage and 98.87% sequence identity to multiple reference strains of *A. baumannii* (e.g., OP985313.1, OP985312.1, and OP985311.1) (Table 1). Although this high similarity value superficially exceeds the standard 98.7% threshold generally accepted for species-level bacterial identification, the 16S rRNA gene is well-known to lack sufficient resolution to definitively differentiate closely related species within the genus *Acinetobacter* (de Souza et al., 2025). Therefore, the BLASTn match was considered indicative only of genus-level assignment to *Acinetobacter sp.*, necessitating comprehensive molecular phylogenetic tree reconstruction to accurately resolve its true taxonomic boundaries and evolutionary lineage.

The other two isolates were also successfully amplified and sequenced and identified as an *Enterobacter sp.* and a *Pseudomonas sp.*; however, their detailed taxonomic identification and phylogenetic placement will be presented in a separate report focusing on broader diversity. The obtained 16S rRNA gene sequences of all three isolate were deposited in the NCBI GenBank database under accession numbers [PZ542785-PZ542787].

Table 1. BLASTn analysis of the nearly complete 16S rRNA gene sequence (1,411 bp) from fluoroquinolone-resistant Isolate 2.

No	Description	Acc. Num	Query Cover (%)	Seq. Iden (%)	E-value
1	A. baumannii strain NG-EB-FETHAb-MW13	OP985313.1	100	98.87	0.00
2	A. baumannii strain NG-EB-FETHAb-MW12	OP985312.1	100	98.87	0.00
3	A. baumannii strain NG-EB-MMHAf-AE11	OP985311.1	100	98.87	0.00
4	Uncultured bacterium clone P6D1-455	EF510278.1	100	98.87	0.00
5	A. baumannii strain AB2041	CP199964.1	100	98.80	0.00
6	A. baumannii strain AB2012	CP199979.1	100	98.80	0.00

Phylogenetic Analysis of Fluoroquinolone-Resistant Isolates

To determine the evolutionary relationships and precise taxonomic position of the novel isolate 2, a Maximum Likelihood phylogenetic tree was constructed based on 16S rRNA gene sequences using RAxML with 500 bootstrap replicates (Fig 4). The tree was robustly rooted using *E. coli* K12, *P. aeruginosa* ATCC 27853, and *P. aeruginosa* PAO1 as outgroups, which formed a well-separated, distinct monophyletic cluster with a bootstrap support value of 97%.

The phylogenetic analysis confirmed the strict monophyly of the genus *Acinetobacter*, resolving it from the outgroup taxa with maximum statistical confidence (97% bootstrap support). Within the *Acinetobacter* lineage, the genus split into distinct subclades, separating the more distantly related species such as *Acinetobacter lwoffii* AB881 (97% support), *Acinetobacter haemolyticus* HW-2 (71% support), and *Acinetobacter johnsonii* EJD.2bj (59% support) from the core clinical complexes.

Strains belonging to *A. baumannii* (ATCC 17978, SDF, AYE, CIP 70.10, ATCC 19606, AB2041, BJAB0715, and AB5075) clustered together into a highly cohesive, tightly resolved monophyletic group with 97% bootstrap support. Intraspecific branching within the *A. baumannii* group exhibited lower bootstrap values (ranging from 3% to 71%), highlighting the conserved nature of the 16S rRNA gene sequence among these distinct reference and clinical isolates.

The 16S rRNA topology clearly demonstrates that isolate 2 (represented in green) does not belong to *A. baumannii*. Instead, it occupies a distinct transitional phylogenetic position within the radiation of non-baumannii *Acinetobacter* species, branching as a sister lineage to a clade composed of *A. soli* AB1143 and the core *A. baumannii* complex (supported by a bootstrap value of 62%). Positioned immediately adjacent to key members of the *Acinetobacter calcoaceticus-baumannii* (ACB) complex, including *Acinetobacter nosocomialis* AB1693 and *Acinetobacter pittii* AB1715, the distinct branch lengths and node resolution confirm its unique lineage. Consequently, this sequence was submitted to the NCBI GenBank database under the official taxon designation *Acinetobacter sp.* strain NEY04 with the accession number PZ542786.

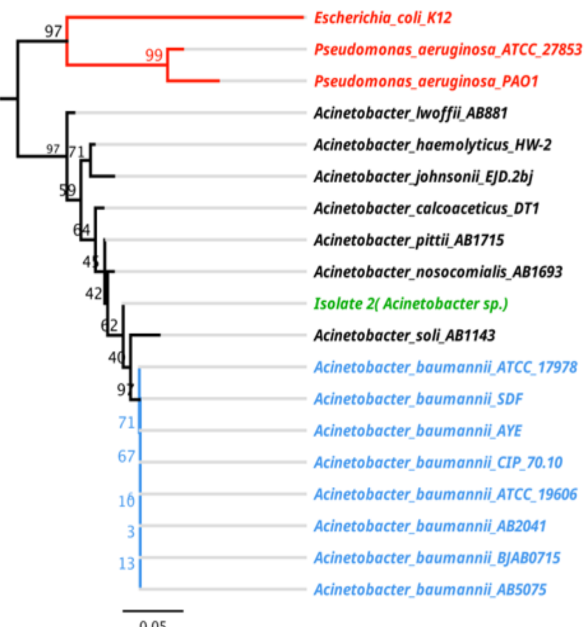

Fig 4. Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequences showing the position of isolate 2 (green) within the genus Acinetobacter. Strains of A. baumannii are highlighted in light blue, while other valid non-baumannii Acinetobacter species are shown in black. E. coli and P. aeruginosa strains served as outgroups (red). Numbers at nodes indicate bootstrap support values (%) based on 500 replicates; values below 50% represent lower topological confidence. The scale bar represents 0.05 substitutions per nucleotide position.

Discussion

Agricultural Selective Pressures and the Emergence of High-Level Quinolone Resistance

The isolation of Acinetobacter sp. strain NEY04, which displays a high-level ciprofloxacin resistance phenotype with an MIC of 35 µg/mL from agricultural fields in Khorasan Razavi Province, highlights the profound impact of anthropogenically driven selective pressures. In poultry production across Iran, fluoroquinolones such as enrofloxacin are frequently administered to livestock, and a substantial portion of these synthetic agents is excreted in active form through feces into poultry manure (Boxall, 2008). The long-term application of this untreated or partially composted litter to fields provides a continuous selective pressure that enriches fluoroquinolone-resistant opportunistic pathogens within the topsoil layer (Wang et al., 2025). While the low-level resistance (MIC ≈ 1 µg/mL) observed in the co-isolated *Enterobacter sp.* and *Pseudomonas sp.* may reflect lower-grade or intrinsic adaptive mechanisms, the pronounced MIC of strain NEY04 points toward highly consolidated, stable resistance mechanisms persisting within the agricultural matrix.

Genomic Plasticity and the Central Role of Acinetobacter in Horizontal Gene Transfer

The taxonomic assignment of Isolate 2 within the genus *Acinetobacter* carries major implications for how antimicrobial resistance spreads across ecosystems. Environmental bacteria exposed to persistent antibiotic residues in manure-impacted soils possess high genomic plasticity, allowing them to capture and spread resistance traits via HGT. Given the critical role of this genus in the horizontal gene transfer of antibiotic resistance genes, these findings provide a vital foundation for better future understanding of the precise mechanisms driving ciprofloxacin resistance within environmental *Acinetobacter* lineages (Hu et al., 2021).

Phylogenetic Resolution as a Foundation for Deciphering Specific Resistance Mechanisms

Our molecular analysis highlights a critical paradox between local sequence identity thresholds and global phylogenetic tree topology. While preliminary BLASTn screening returned a 98.87% identity to multiple reference strains of *A. baumannii*, the hyper-conserved nature of the 16S rRNA gene within this genus prevents reliable species-level identification using similarity scores alone (de Souza et al., 2025). The Maximum Likelihood phylogenetic tree reconstructed using

RAxML successfully resolved these boundaries, demonstrating that Isolate 2 does not belong to the strict *A. baumannii* monophyletic clade. Instead, it branches as a distinct transitional sister lineage to *A. soli* and the core *A. baumannii* complex, positioned adjacent to non-baumannii clinical species like *A. nosocomialis* and *A. pittii*.

Accurately outlining this unique lineage is an essential prerequisite for downstream mechanistic studies. Because ciprofloxacin resistance mechanisms such as mutations in the quinolone resistance-determining regions (QRDR) of gyrA and parC, or the mobilization of plasmid-mediated quinolone resistance (PMQR) genes can vary across species boundaries (Espinal et al., 2011), having a clear evolutionary framework ensures that future genetic analyses are properly contextualized within environmental non-baumannii architectures rather than clinical templates.

Limitations and Future Directions

The primary limitation of this study lies within its baseline molecular scope relying exclusively on the hyper-conserved 16S rRNA gene marker provided insufficient taxonomic resolution to resolve the precise species-level boundary of *Acinetobacter sp.* strain NEY04, leaving it classified at the genus level. Furthermore, while the study successfully established a high-level ciprofloxacin resistance phenotype, it remains limited by the lack of direct genotypic verification of the underlying resistance mechanisms. To resolve these limitations, immediate future directions will focus on sequencing the specific genetic determinants responsible for this advanced antimicrobial resistance.

Conclusion

This study successfully isolated and characterized a highly ciprofloxacin-resistant bacterium, *Acinetobacter sp.* strain NEY04 (MIC = 35 μg/mL), from poultry manure-amended agricultural soils in Khorasan Razavi Province, Iran. While local sequence similarity scores initially suggested an assignment of *A. baumannii*, robust Maximum Likelihood phylogenetic tree reconstruction demonstrated that the isolate instead occupies a distinct, transitional non-baumannii lineage. This taxonomic discrepancy highlights the clear limitations of relying solely on basic BLASTn percentage thresholds for identifying species within hyper-conserved bacterial complexes. Ultimately, because of the prominent role the genus *Acinetobacter* plays in the horizontal gene transfer of resistance determinants, these findings provide a vital evolutionary framework. This work establishes a necessary baseline for future down-stream sequencing of specific genetic mechanisms such as QRDR mutations or PMQR elements driving advanced fluoroquinolone resistance in environmental agro-ecosystems.

Ethical Statement

This study does not involve any human participants, clinical trials, or vertebrate animal experimentation. Soil samples were collected from commercial agricultural fields with strict compliance to standard environmental research practices, and the specific names of the source farms were anonymized to maintain confidentiality.

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