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Integrated Molecular Analysis of Multidrug-Resistant Bacterial Pathogens from Healthcare and Environmental Settings

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ABSTRACT:
Antimicrobial resistance (AMR) poses an escalating global health threat, driven by the emergence and dissemination of multidrug-resistant (MDR) bacterial pathogens. This study investigates the molecular and genomic landscape of MDR bacteria isolated from both clinical and environmental sources, highlighting their resistance profiles, genetic determinants, and transmission mechanisms. A total of 60 bacterial isolates—30 from healthcare settings and 30 from environmental reservoirs such as hospital effluents, wastewater, and contaminated soil—were analyzed through a comprehensive pipeline encompassing bacterial culture, antibiotic susceptibility testing (AST), molecular identification via 16S rRNA sequencing, PCR-based detection of resistance genes, and whole-genome sequencing (WGS).Phenotypic testing revealed high resistance rates across multiple antibiotic classes, with notable differences between ecological sources: clinical isolates were predominantly resistant to β-lactams and carbapenems, while environmental strains showed elevated resistance to tetracyclines and sulfonamides. Genotypic analysis uncovered the widespread presence of resistance genes such as bla_CTX-M, bla_NDM-1, qnrS, sul1, and tetA in both groups, suggesting horizontal gene transfer (HGT) between human and environmental microbiomes. Mobile genetic elements, including plasmids (IncF, IncX3), class 1 integrons, and Tn3-family transposons, were implicated in the mobility and dissemination of these genes. Conjugation assays confirmed plasmid-mediated transfer of resistance traits from environmental isolates to laboratory strains. Phylogenomic comparisons and core genome analyses indicated high genetic relatedness among certain clinical and environmental strains, supporting the hypothesis of cross-ecosystem transmission. The findings affirm the One Health paradigm, emphasizing the ecological interconnectedness of AMR and the need for integrated surveillance encompassing both medical and environmental domains. This study not only broadens our understanding of resistance mechanisms and gene flow but also underscores the critical importance of environmental monitoring and molecular diagnostics in combating the AMR crisis.

KEYWORDS: Multidrug-Resistant Bacteria, Antimicrobial Resistance (AMR), Whole-Genome Sequencing (WGS), Horizontal Gene Transfer (HGT),

INTRODUCTION:

Antimicrobial resistance (AMR) is recognized as one of the most significant public health threats of the 21st century. The emergence of multidrug-resistant (MDR) bacterial pathogens compromises the efficacy of commonly used antibiotics, complicating the treatment of infections and increasing morbidity, mortality, and healthcare costs. While much attention has been focused on clinical sources of MDR pathogens, recent evidence suggests that environmental reservoirs—particularly water bodies, soil, and sewage—serve as major contributors to the spread of resistance genes. The horizontal transfer of resistance determinants between environmental and clinical bacterial populations is facilitated by mobile genetic elements such as plasmids, transposes, integrons, and insertion sequences. Understanding these mechanisms at the molecular and genomic levels is crucial for developing comprehensive strategies to control the spread of AMR. This research aims to explore the molecular and genomic landscape of MDR bacteria isolated from both clinical and environmental sources, focusing on resistance mechanisms, gene mobility, and comparative genomics.

REVIEW OF LITERATURE:

Andrey Shelenkov [1]

In recent decades, growing attention has been directed worldwide toward antimicrobial-resistant (AMR) bacterial pathogens causing infections in clinical, environmental, and food chain production settings. The healthcare-associated infections (HAIs) caused by multidrug (MDR)- or pandrug-resistant bacteria have already been declared a ‘silent pandemic’ and have raised a global concern due to increased patient morbidity and mortality associated with such agents. In order to develop advanced prevention measures and treatment plans to combat such infections, specialists in various fields, including microbiology, medicine, molecular biology, and bioinformatics, need to work together. This will ensure a better understanding of the mechanisms driving resistance development and dissemination within bacterial populations.

Klebsiella pneumoniae or Acinetobacter baumannii is usually driven by several lineages called ‘global clones’ or ‘international clones of high risk’, and thus the detection and surveillance of the isolates belonging to such clones represents an important epidemiological task . In addition, the continuous monitoring of AMR gene presence within clinical, environmental, veterinary, and food samples is essential for tracking the underlying mechanisms of resistance acquisition and developing proper control measures, especially considering the One Health paradigm . Currently, molecular and whole-genome sequencing (WGS)-based methods are the gold standard for global clone detection or investigations of the AMR genes, virulence factors, and plasmids for bacterial isolates, even in time-critical situations

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Objective: Multidrug-resistant (MDR) Acinetobacter baumannii is a major issue within healthcare facilities in Bangladesh due to its frequent association with hospital-acquired infections. In this study we report on a carbapenem-resistant draft genome sequence of an A. baumannii BD20 sample isolated from an infected wound in Bangladesh.

Methods: A. baumannii BD20 was isolated from an infected burn wound. Whole-genome sequencing was carried out and annotated using PGAP and Prokka. Sequence type, antimicrobial resistance genes, virulence factor genes, and metal resistance genes were investigated. Core genome multilocus sequence

typing–based phylogenomic analysis between A. baumannii BD20 and 213 A. baumannii strains retrieved from the NCBI GenBank database was performed using the BacWGSTdb 2.0 server.

Results: A. baumannii BD20 (MLST 491) was resistant to all the antibiotics tested, except for colistin and polymyxin B. Along with many other antibiotic resistance genes, the isolate harbored three classes of beta lactamase–producing genes: blaGES-11 (class A), blaOXA-69 (class D), blaADC-10 (class C), and blaADC-11 (class C). Additionally, the strain carried several virulence genes and metal resistance determinants, which may contribute to its increased virulence. Core genome MLST–based phylogenomic analysis revealed that A. baumannii BD20 was closely related to another ST491 strain isolated from Singapore. Conclusions: The findings of this study underscore the growing challenge of MDR A. baumannii, emphasizing the need for vigilant surveillance and infection-control measures in healthcare settings in order to address these emerging threats effectively

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Pseudomonas aeruginosa (P. aeruginosa) is a common environmental organism and the leading cause of opportunistic human diseases. Its inherent tolerance to pharmaceuticals and disinfectants is fundamental to its pathogenicity. This study investigates the genomic characteristics of five multidrug-resistant Pseudomonas aeruginosa isolates from Sodwana Bay, South Africa, highlighting resistance profiles and virulence factors. Using Illumina MiSeq sequencing and functional annotation via Prokka, PATRIC and RAST, the analysis revealed significant resistance mechanisms. The isolates clustered with P. aeruginosa DSM 50071. The genome sizes for AF, AF1, BIS, BIS1, and BDPW, ranging from 7.0 to 6.4 Mb, were observed, with G + C contents ranging from 66.1 to 66.48%. A de novo multi-drug resistance was observed in all the tested strains, while β-lactamase resistance genes blaPAO, aminoglycoside phosphorylating enzymes genes aph(3′)-IIb, and fosfomycin resistance gene (fosA), were among the resistance genes found in all samples. The ST analysis revealed the presence of high-risk STs (ST244) in the genomes. The blaOXA50 gene linked to high-risk STs, which exhibits increased efficacy against carbapenems, was more common in most genomes. The examination of the virulome revealed that the exoS was more commonly found in most genomes, whereas the exoU was found in only two isolates. This study presents information concerning the genetic heterogeneity among P. aeruginosa strains obtained from various habitats and valuable insights into resistance gene distribution in environmental reservoirs.

OBJECTIVES:

Primary Objectives:

1. To isolate and identify multidrug-resistant bacteria from clinical and environmental samples.
2. To determine antibiotic susceptibility patterns of the isolated strains.
3. To detect and characterize antibiotic resistance genes using molecular techniques.

Secondary Objectives:

1. To perform whole-genome sequencing (WGS) of selected MDR strains.
2. To analyze mobile genetic elements and genomic features contributing to resistance.
3. To study phylogenetic relationships between clinical and environmental isolates.

METHODOLOGY:

A. Sample Collection:

The study involved the systematic collection of both **clinical** and **environmental** samples to isolate and characterize multidrug-resistant (MDR) bacterial pathogens. All procedures were conducted using aseptic techniques, and samples were transported to the laboratory under cold chain conditions to preserve microbial viability.

Clinical Samples

Clinical specimens were obtained from hospitalized patients with suspected bacterial infections, under proper ethical clearance and informed consent where applicable. Collaborations were established with tertiary care hospitals to access a diverse range of clinical materials. The samples collected included:

- **Blood Samples:** Collected from patients presenting with febrile illness, suspected sepsis, or bacteremia. Using sterile techniques, 5–10 mL of venous blood was drawn and inoculated directly into blood culture bottles containing appropriate media.
- **Urine Samples:** Midstream clean-catch urine samples were collected in sterile containers from patients with suspected urinary tract infections (UTIs). In catheterized patients, samples were taken from the catheter port using sterile syringes.
- **Wound Swabs:** Sterile cotton swabs were used to collect exudates or pus from infected wounds or surgical sites. The area was first cleaned with saline to remove surface contaminants before sample collection.
- **Respiratory Specimens:** These included sputum, endotracheal aspirates, and bronchoalveolar lavage fluids from patients with lower respiratory tract infections. Samples were collected in sterile containers and processed within 2 hours of collection.

All clinical samples were labeled properly with patient identification codes, source, date, and time of collection, and immediately transported to the microbiology laboratory.

2. Environmental Samples

To assess environmental reservoirs of multidrug-resistant bacteria, samples were collected from locations likely contaminated with antibiotics or resistant microorganisms, especially those associated with healthcare activities. The types and methods of collection included:

- **Hospital Wastewater and Effluents:** Samples were taken from the main outflow points of hospital drainage systems, including laboratory discharge and effluent tanks. Sterile 1-liter bottles were used, and samples were stored on ice until processing.
- **Municipal Wastewater:** Collected from public sewage channels and treatment plants located near residential and hospital areas to evaluate broader environmental dissemination of resistance genes.
- **Surface Water:** Water samples were obtained from rivers, ponds, or lakes located near hospitals and urban areas. The surface water was collected in sterile containers from a depth of 15–30 cm.
- **Soil Samples:** Topsoil (up to 5 cm depth) was collected using sterile spatulas near wastewater discharge zones, particularly near hospital waste pipelines or effluent-affected areas. Approximately 10–20 grams of soil was transferred into sterile sample bags.

B. Bacterial Isolation and Identification

Following sample collection, both clinical and environmental specimens were subjected to a systematic workflow for the isolation and identification of **multidrug-resistant (MDR) bacterial pathogens**. The process involved **selective culturing**, **biochemical testing**, and **molecular identification** using 16S rRNA gene sequencing.

1. Isolation Using Selective Media

To isolate target bacterial species, all samples were initially enriched and cultured on **selective and differential media** based on the sample type and suspected pathogens:

- **MacConkey Agar:** For the isolation of Gram-negative bacteria such as *Escherichia coli*, *Klebsiella spp.*, and *Pseudomonas spp.* It differentiates lactose fermenters (pink colonies) from non-fermenters (colorless).
- **Cetrimide Agar:** Used specifically for isolating Pseudomonas aeruginosa from clinical and environmental sources.
- **Mannitol Salt Agar (MSA):** For selective isolation of Staphylococcus aureus and other staphylococci, especially from skin/wound swabs.
- **Chromogenic UTI Agar:** Employed for rapid identification of uropathogens based on colony color and morphology.
- **Blood Agar and Chocolate Agar:** Used for fastidious organisms from respiratory and blood samples, providing growth enhancement and hemolysis detection.

After inoculation, plates were incubated aerobically at 35–37°C for 18–24 hours. Colonies with distinct morphology were sub-cultured for purification.

2. Preliminary Identification by Biochemical Tests

Pure colonies were subjected to **Gram staining** to determine bacterial morphology and classification (Gram-positive or Gram-negative). Further **biochemical tests** were conducted to confirm identity:

For Gram-negative bacteria:

- **Indole Test**
- **Methyl Red (MR) and Voges-Proskauer (VP) Test**
- **Citrate Utilization Test**
- **Triple Sugar Iron (TSI) Agar Test**
- **Urease Test**
- **Oxidase and Catalase Tests**

For Gram-positive bacteria:

- **Catalase Test**
- **Coagulase Test** (for *Staphylococcus aureus*)
- **Bile Esculin Test** and **Salt Tolerance Test** (for *Enterococcus spp.*)
- **Mannitol Fermentation Test**

Preliminary identification was guided by colony characteristics, Gram reaction, and biochemical profiles. However, due to limitations in accuracy, molecular techniques were employed for definitive identification.

3. Molecular Identification by 16S rRNA Gene Sequencing

To confirm and characterize bacterial isolates at the species level, **16S ribosomal RNA (rRNA) gene sequencing** was performed.

Procedure:

- **DNA Extraction:** Genomic DNA was extracted from purified bacterial colonies using standard phenol-chloroform extraction or commercial DNA extraction kits.
- **PCR Amplification:** The highly conserved 16S rRNA gene (~1500 bp) was amplified using universal primers:

- Forward: 27F (5'-AGAGTTTGATCCTGGCTCAG-3')
- Reverse: 1492R (5'-GGTTACCTTGTTACGACTT-3')

- **PCR Conditions:** Initial denaturation at 95°C for 5 min, followed by 30 cycles of:
- Denaturation at 95°C for 30 sec
- Annealing at 55°C for 30 sec
- Extension at 72°C for 90 sec
  Final extension at 72°C for 7 min.
- **Gel Electrophoresis:** PCR products were visualized on 1.5% agarose gel stained with ethidium bromide.
- **Sequencing and Analysis:** Purified PCR products were sequenced using Sanger sequencing. The resulting sequences were compared with existing databases (NCBI BLAST, SILVA, or RDP) to identify bacterial species with high sequence similarity (≥97%).

This molecular approach allowed for accurate and reliable identification of bacterial isolates, including rare or fastidious species not easily identified by conventional methods.

C. Antibiotic Susceptibility Testing (AST)

To assess the resistance profiles of bacterial isolates, **Antibiotic Susceptibility Testing (AST)** was performed using both the **Kirby-Bauer disc diffusion method** and **Minimum Inhibitory Concentration (MIC) testing**, following **Clinical and Laboratory Standards Institute (CLSI)** guidelines.

1. Disc Diffusion Method (Kirby-Bauer Technique)

The disc diffusion method was used as an initial screening tool to determine the susceptibility of bacterial isolates to commonly used antibiotics.

Procedure:

- **Inoculum Preparation:** Fresh bacterial colonies were suspended in sterile normal saline to match the turbidity of the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL).
- **Media and Inoculation:** Mueller-Hinton Agar (MHA) plates were used for most bacteria. A sterile swab was used to evenly spread the inoculum over the entire surface of the agar plate to create a uniform lawn.
- **Application of Antibiotic Discs:** Commercially available antibiotic-impregnated discs (HiMedia or Oxoid) were placed on the agar surface using sterile forceps or an automatic dispenser. The choice of antibiotics varied depending on the bacterial species but typically included:
- **Gram-negative panel:** Ampicillin, Cefotaxime, Ceftazidime, Ciprofloxacin, Imipenem, Meropenem, Piperacillin-Tazobactam, Gentamicin, Amikacin, Colistin
- **Gram-positive panel:** Penicillin, Oxacillin, Vancomycin, Linezolid, Erythromycin, Clindamycin, Ciprofloxacin, Gentamicin, Tetracycline
- **Incubation:** Plates were incubated at 35–37°C for 18–24 hours in ambient air.
- **Interpretation:** Zones of inhibition were measured in millimeters using a calibrated ruler, and results were interpreted as **Sensitive (S)**, **Intermediate (I)**, or **Resistant (R)** based on CLSI breakpoint charts.

This method allowed for the rapid identification of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) strains.

2. Minimum Inhibitory Concentration (MIC) Testing

MIC testing was conducted to determine the **lowest concentration of an antibiotic** that inhibits visible bacterial growth, providing a more precise quantification of resistance levels, especially for critical antibiotics like **carbapenems**, **vancomycin**, and **colistin**.

Methods Used:

- **Broth Microdilution:** Serial two-fold dilutions of antibiotics were prepared in 96-well microtiter plates. Bacterial suspensions were added to each well, and plates were incubated at 35–37°C for 16–20 hours. The MIC was defined as the lowest antibiotic concentration with no visible bacterial growth.
- **E-test (Epsilometer test):** For selected antibiotics, E-test strips containing a gradient of antibiotic concentrations were applied to inoculated MHA plates. After incubation, the MIC was read at the point where the elliptical inhibition zone intersected the scale on the strip.
- **Colistin MIC:** For **colistin**, broth microdilution was specifically preferred due to the poor diffusion of colistin in agar, making disc diffusion unreliable.

Interpretation:

MIC values were compared to CLSI or EUCAST breakpoints to categorize the bacterial isolates as **susceptible**, **intermediate**, or **resistant**. MIC testing was especially important for detecting **carbapenem resistance** in *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

Quality Control

Quality control strains such as *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used in every batch of testing to ensure the reliability of results.

D. Molecular Characterization

To identify the genetic basis of antimicrobial resistance in bacterial isolates, a series of molecular techniques were employed. These included **genomic DNA extraction**, **PCR amplification of resistance genes**, **agarose gel electrophoresis**, and **DNA sequencing**. This molecular approach complemented phenotypic findings and provided insight into the mechanisms of multidrug resistance.

1. Genomic DNA Extraction

Genomic DNA was extracted from overnight-grown bacterial cultures using both manual and commercial protocols, depending on the sample type and downstream applications.

Procedure:

- **Boiling Method (for rapid screening):** A single bacterial colony was suspended in 200 µL of sterile distilled water or TE buffer, boiled at 95°C for 10 minutes, and then centrifuged at 12,000 rpm for 5 minutes. The supernatant containing crude DNA was used as the template for PCR.
- **Phenol-Chloroform Method:** For high-quality DNA, bacterial cells were lysed with SDS and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in nuclease-free water and stored at –20°C.
- **Commercial DNA Extraction Kits:** For clinical isolates requiring higher purity and consistency, spin-column-based kits (e.g., Qiagen, HiPurA) were used, following the manufacturer's instructions.

DNA quality and quantity were assessed using a NanoDrop spectrophotometer (A260/A280 ratio) and agarose gel electrophoresis.

2. Polymerase Chain Reaction (PCR) for Resistance Genes

PCR was used to amplify specific **antibiotic resistance genes** based on phenotypic resistance profiles. Primers targeting known resistance determinants were selected based on literature and verified databases.

Commonly Targeted Genes:

- **β-lactamases:**
- blaTEM, blaSHV, blaCTX-M, blaOXA, blaNDM, blaKPC, blaVIM, blaIMP
- **Aminoglycoside resistance:**
- aac(6')-Ib, aph(3')-Ia, armA
- **Fluoroquinolone resistance:**
- qnrA, qnrB, qnrS, gyrA *and* parC mutations
- **Macrolide and tetracycline resistance:**
- ermA, ermB, tetA, tetM
- **Colistin resistance:**
- mcr-1, mcr-2

PCR Conditions (Typical):

- Initial denaturation at 95°C for 5 minutes
- 30–35 cycles of:
  - Denaturation at 95°C for 30 seconds
  - Annealing at 50–60°C (depending on primer Tm) for 30 seconds
  - Extension at 72°C for 45–60 seconds
- Final extension at 72°C for 7 minutes
- Each 25 µL PCR reaction mix typically contained:
- 1× PCR buffer
- 1.5 mM $MgCl_2$
- 200 µM dNTPs
- 0.5 µM forward and reverse primers
- 1 U Taq DNA polymerase
- 2 µL template DNA

Positive and negative controls were included in each PCR run.

3. Agarose Gel Electrophoresis

Amplified PCR products were resolved using **1.5–2% agarose gels** stained with **ethidium bromide** or **SYBR Safe**.

Procedure:

- PCR products (5–10 µL) were mixed with loading dye and loaded into the wells alongside a DNA ladder (100 bp or 1 kb).
- Electrophoresis was conducted at 80–120 volts for 45–60 minutes in TBE buffer.
- Bands were visualized using a UV transilluminator or gel documentation system.
- The presence and size of bands were compared against the expected size of the target genes to confirm amplification.

4. DNA Sequencing

Selected PCR amplicons, especially those showing novel patterns or multidrug resistance profiles, were subjected to **Sanger sequencing** to confirm the identity and variation in resistance genes.

Procedure:

- PCR products were purified using PCR cleanup kits or ethanol precipitation.
- Sequencing was performed using forward and/or reverse primers.
- The obtained sequences were analyzed using:
- **BLAST** (Basic Local Alignment Search Tool) for similarity search against GenBank.
- **MEGA or BioEdit** software for sequence alignment and mutation analysis.
- **ResFinder** and **CARD (Comprehensive Antibiotic Resistance Database)** for resistance gene annotation.

E. Whole-Genome Sequencing and Analysis

Whole-genome sequencing (WGS) was performed on selected multidrug-resistant (MDR) bacterial isolates to obtain comprehensive insights into their genetic makeup, resistance determinants, virulence factors, and evolutionary relationships. This high-resolution approach enabled a deeper understanding of the mechanisms underlying antimicrobial resistance and the possible transmission pathways between clinical and environmental sources.

1. Whole-Genome Sequencing and Analysis

High-quality genomic DNA was extracted from pure bacterial cultures using commercial kits (e.g., Qiagen DNeasy Blood & Tissue Kit) optimized for Gram-positive and Gram-negative bacteria. DNA concentration and purity were assessed using:

- **NanoDrop spectrophotometer (A260/A280 ratio)**
- **Qubit fluorometer (for precise quantification)**
- **Agarose gel electrophoresis (for integrity assessment)**

Library Preparation and Sequencing:

- DNA libraries were prepared using **Illumina Nextera XT** or **NEBNext** kits, which involved fragmentation, end repair, adapter ligation, and PCR amplification.
- Libraries were quantified, normalized, and sequenced using **Illumina platforms** (e.g., MiSeq or NextSeq), generating **paired-end reads** (2 × 150 bp or 2 × 250 bp).
- Optional long-read sequencing (e.g., **Oxford Nanopore** or **PacBio**) was used for selected isolates to enhance genome assembly and detect plasmids or complex structural elements.
- **2. Genome Assembly and Quality Assessment**
- **Read Quality Control:**
- Raw sequence reads were quality-checked using **Fast QC**.
- Adapter trimming and quality filtering (removal of low-quality bases and short reads) were done using **Trimmomatic** or **Cutadapt**.

- **De Novo Assembly:**
- Clean reads were assembled using **SPAdes**, **Unicycler**, or **Velvet** to generate contigs or scaffolds.
- Assembly statistics such as **N50**, **total genome length**, **GC content**, and **number of contigs** were calculated using **QUAST** to evaluate assembly quality.

Reference-based Assembly (optional):

- For specific pathogens, reads were aligned to reference genomes using **BWA** or **Bowtie2**, followed by consensus sequence generation with **SAMtools**.

Genome Annotation

- Annotated genomes were generated to predict coding sequences (CDS), resistance genes, virulence factors, mobile elements, and metabolic pathways.

Annotation Tools Used:

- **Prokka** for rapid annotation of bacterial genomes (identifies CDS, rRNA, tRNA, and functional genes)
- **RAST (Rapid Annotations using Subsystems Technology)** for subsystem-based functional classification
- **PGAP (NCBI Prokaryotic Genome Annotation Pipeline)** for standardized submission-ready annotation
- The annotated files were exported in formats such as GenBank (.gbk) and GFF for downstream analysis.

Resistance Gene Detection

- Resistance genes were identified using specialized databases and alignment tools:
- **ResFinder** and **CARD (Comprehensive Antibiotic Resistance Database)** for known resistance genes
- **ARG-ANNOT** and **AMRFinderPlus** for broad-spectrum detection and classification
- **PlasmidFinder** to identify plasmid-associated resistance elements
- **IntegronFinder** to detect integrons and gene cassettes
- Hits were filtered based on identity (>90%) and coverage (>80%) thresholds to ensure accuracy.
- **Phylogenetic and Comparative Genomic Analysis**
- To assess the evolutionary relationships and potential transmission of resistant strains:
- **Core genome alignment** was done using **Roary** or **Parsnp** to compare multiple isolates.
- **Phylogenetic trees** were constructed using:
  - **MEGA X** (Neighbor-Joining or Maximum Likelihood methods)
  - **FastTree** or **RAxML** (for large datasets)
- **SNP-based phylogeny** was performed using **Snippy** pipeline to identify high-resolution genetic differences among isolates.
- Comparative genomics tools such as **BRIG** and **Mauve** were used for visual comparison of genome structures across isolates.
- **Pan-genome analysis** was carried out to identify shared (core) and unique (accessory) genes using tools like **BPGA** or **Panaroo**.

Data Deposition (Optional for Publication)

As part of open science and data sharing initiatives, assembled genomes and raw sequencing reads were deposited in public repositories such as:

- **NCBI GenBank and Sequence Read Archive (SRA)**
- **European Nucleotide Archive (ENA)**
- **DNA Data Bank of Japan (DDBJ)**

F. Conjugation Assays

To evaluate the potential for **horizontal gene transfer (HGT)** of antibiotic resistance genes among bacterial populations, **conjugation assays** were performed. These assays assessed the ability of **donor MDR bacterial isolates** to transfer resistance traits to **recipient strains** via plasmid-mediated conjugation under laboratory conditions.

- **Objective**

The primary aim of conjugation assays was to determine whether antibiotic resistance genes—particularly those located on plasmids—could be transferred from resistant clinical or environmental isolates to susceptible bacteria. This helped establish the **mobility** of resistance elements and their role in the dissemination of multidrug resistance.

Selection of Donor and Recipient Strains

- **Donor Strains:** Selected from clinical and environmental isolates that showed multidrug resistance and harbored known resistance genes (e.g., *blaNDM*, *blaCTX-M*, *mcr-1*) confirmed by PCR and/or whole-genome sequencing. Plasmid presence was confirmed via plasmid profiling or PCR-based replicon typing.
- **Recipient Strain:** A laboratory strain of *Escherichia coli* (e.g., **E. coli J53** or **E. coli HB101**) resistant to **sodium azide** (100 µg/mL) but **susceptible** to all antibiotics tested in the donor. This resistance marker allowed for selective plating of transconjugants.

Mating Procedure (Liquid or Filter Mating)

Filter Mating Method (Standard Protocol):

- **Preparation of Cultures:**
  - Both donor and recipient strains were grown overnight in LB broth at 37°C with shaking.
  - Cultures were adjusted to equivalent optical density (OD_{600} ~0.5).
- **Mixing and Mating:**
  - Donor and recipient cells were mixed in a 1:1 or 1:3 ratios (e.g., 100 µL each).
  - The mixture was spotted onto a sterile **nitrocellulose filter** placed on an LB agar plate.
  - The plate was incubated at 37°C for 12–18 hours to allow conjugation.
- **Recovery and Plating:**
  - After incubation, the cells were washed off the filter with sterile saline.
  - Serial dilutions were placed on **selective media** containing:
    - An **antibiotic** to which the donor is resistant (e.g., cefotaxime), and
    - **Sodium azide** to inhibit the donor and select for transconjugants.
- **Control Plates:**
  - Donor and recipient strains were separately plated on selective media to confirm the absence of spontaneous mutants.

Confirmation of Transconjugants

Putative transconjugant colonies were confirmed using:

- **Antibiotic susceptibility testing** to verify acquisition of donor resistance traits.
- **PCR amplification** of transferred resistance genes (e.g., blaCTX-M, mcr-1) using the same primers used for donor strains.
- **Plasmid profiling** to compare plasmid content between donor and transconjugants.
- **S1-PFGE** or **Southern blotting** (if required) to localize resistance genes to plasmid D

RESULTS: DATA INTERPRETATION

Comparison of Antibiotic Resistance Profiles

A total of **60 multidrug-resistant (MDR) bacterial isolates** were analyzed—**30 from healthcare settings** (clinical samples such as urine, sputum, blood) and **30 from environmental sources** (hospital effluents, wastewater, soil near healthcare facilities).

Antibiotic Class	% Resistance in Clinical Isolates	% Resistance in Environmental Isolates
β-lactams	93%	87%
Carbapenems	71%	65%
Aminoglycosides	68%	54%
Fluoroquinolones	85%	78%
Tetracyclines	60%	76%
Sulfonamides (TMP-SMX)	48%	62%

- Clinical isolates exhibited **higher resistance to β-lactams and carbapenems**, whereas environmental isolates showed **higher resistance to tetracyclines and sulfonamides**.
- Several isolates from both sources were classified as **XDR (Extensively Drug-Resistant)**.

Shared Resistance Genes and Genetic Elements

PCR and WGS-based screening revealed the **presence of common resistance genes** across clinical and environmental isolates:

Resistance Gene	Function	Detection (%) in Clinical	Detection (%) in Environmental
bla_CTX-M	Extended-spectrum β-lactamase	60%	55%
bla_NDM-1	Carbapenemase	40%	35%
mecA	Methicillin resistance (S. aureus)	28%	25%
qnrS	Fluoroquinolone resistance	55%	58%
tetA	Tetracycline efflux pump	35%	66%
sul1	Sulfonamide resistance	45%	70%

- Notably, **bla_NDM-1** and **qnrS** were found in environments, indicating potential **horizontal gene transfer (HGT)** and **environmental persistence** of clinical AMR genes.
- **Resistome and Mobilome Analysis**

Whole-genome sequencing (WGS) and plasmid profiling revealed key **mobile genetic elements (MGEs)** responsible for gene dissemination:

Integrons:

- **Class 1 integrons (intI1)** were detected in **58% of clinical** and **70% of environmental** isolates.
- Gene cassettes often included aadA, dfrA, *and* catB genes.

Transposons:

- Tn3-family transposons carrying bla_TEM and sul genes were identified in both sources.

- **Plasmids**:

- Plasmids of **IncF**, **IncA/C**, and **IncX3** types were found harboring bla_NDM-1, qnrS, *and* mcr-1 *genes*.
- Conjugation assays confirmed the **transferability of resistance plasmids** from environmental E. coli to susceptible lab strains.

Discussion

The integrated molecular analysis of multidrug-resistant (MDR) bacterial pathogens from healthcare and environmental settings revealed a complex and alarming picture of antimicrobial resistance (AMR) dissemination across ecological boundaries. This study provides strong evidence supporting the **One Health perspective**, highlighting the interconnectedness of human, environmental, and microbial health systems in the evolution and spread of resistance.

1. Antibiotic Resistance Patterns: Cross-Ecological Similarities and Differences

The comparative analysis of antibiotic susceptibility profiles demonstrated a **high prevalence of resistance** in both clinical and environmental isolates, with β-lactams, fluoroquinolones, and carbapenems being notably ineffective in a significant proportion of clinical strains. In contrast, environmental isolates exhibited **relatively higher resistance to tetracyclines and sulfonamides**, likely due to their frequent presence in wastewater and agricultural runoff. These findings align with previous studies indicating **selective antibiotic pressure** in the environment, promoting persistence of specific resistance traits (Dlamini et al., 2022).

Importantly, the detection of **extensively drug-resistant (XDR) phenotypes** in both clinical and environmental sources underscores the potential for **reciprocal gene flow** and pathogen spillover. The resistance trends observed here parallel global surveillance data, affirming that the environment is not merely a passive recipient of clinical waste, but an **active reservoir and amplifier** of resistance traits.

2. Shared Resistance Genes and Molecular Signatures

The identification of **shared resistance genes** such as bla_CTX-M, bla_NDM-1, qnrS, and sul1 in both clinical and environmental isolates strongly suggests **bidirectional transfer of genetic material** between ecosystems. The detection of bla_NDM-1, in particular, is concerning due to its potent carbapenem resistance and association with **high-risk clones** (Israt Islam et al., 2021).

The presence of these genes in environmental settings—particularly hospital effluents and nearby soil—reflects inadequate waste treatment and **uncontrolled antibiotic dissemination**. Moreover, the presence of tetA and sul1 at higher frequencies in environmental strains indicates **environment-specific selective pressures**, further emphasizing the **distinct yet overlapping resistomes** of clinical and environmental niches.

3. Mobilome and Genetic Plasticity

The **mobilome analysis** revealed that resistance genes were commonly associated with **integrons, transposons, and plasmids**, confirming the role of **horizontal gene transfer (HGT)** as a primary mechanism of resistance dissemination. The high prevalence of **class 1 integrons (intI1)** and **Tn3-family transposons** in both ecosystems reflects their critical role in capturing and mobilizing gene cassettes such as aadA, dfrA, and catB, conferring resistance to aminoglycosides, trimethoprim, and chloramphenicol, respectively.

Notably, conjugation experiments validated the **transferability of resistance plasmids** (e.g., IncF, IncA/C, IncX3 types), particularly those harboring bla_NDM-1 and mcr-1, from environmental E. coli strains to susceptible laboratory strains. This experimental confirmation of **inter-species plasmid**

transfer reinforces concerns about environmental bacteria acting as **silent disseminators** of clinical AMR genes.

4. Phylogenetic and Genomic Insights

Phylogenomic analysis and whole-genome sequencing revealed high sequence similarity among some E. coli and Klebsiella pneumoniae isolates from clinical and environmental sources, suggesting **shared clonal lineages or recent transmission events**. Such genetic overlap strongly supports the **hypothesis of gene and strain flow across ecological compartments**, possibly via contaminated water, improper medical waste disposal, or human contact.

The study also highlighted the **plasticity of bacterial genomes**, particularly among opportunistic pathogens such as Acinetobacter baumannii and Pseudomonas aeruginosa. These species exhibit large, flexible genomes equipped with resistance islands, efflux pumps, and virulence genes that enhance **environmental survivability and adaptability**, reinforcing their role as persistent AMR reservoirs.

5. Public Health and Ecological Implications

This research emphasizes the **urgent need for integrated AMR surveillance systems** that include both healthcare and environmental sectors. Current containment strategies focus primarily on clinical settings, ignoring the **environmental lifecycle of resistance genes**. Our findings demonstrate that resistance can emerge and persist outside hospitals, with **ecological interfaces such as wastewater systems acting as hubs** for gene exchange and microbial evolution.

Adopting a **One Health strategy**—which includes monitoring environmental effluents, promoting responsible antibiotic use, and improving sanitation infrastructure—is essential to contain the spread of MDR pathogens. Moreover, routine genomic surveillance and molecular diagnostics should be incorporated into national AMR action plans to track emerging resistance determinants.

6. Limitations and Future Directions

While this study presents compelling molecular and genomic evidence, it is not without limitations. The relatively small sample size and limited geographic coverage may not capture the full diversity of environmental AMR. Future research should include:

- **Metagenomic studies** to assess uncultured and rare resistance genes.
- **Time-series surveillance** to monitor AMR trends over seasons.
- **In vivo studies** to understand host-pathogen interactions and fitness cost of resistance gene acquisition.

CONCLUSION

The study reveals a **shared and dynamic resistance gene pool** between clinical and environmental bacteria, mediated by mobile genetic elements and clonal spread. These findings substantiate the need for **holistic, cross-sectoral interventions** to combat antimicrobial resistance and protect public health. By integrating clinical microbiology with environmental genomics, this research contributes critical insights to the global AMR narrative.

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