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In Vitro Characterization Of Antimicrobial Resistance And Biochemical Signatures Of Pathogens From Environmental Reservoirs In Pakbara, Moradabad, Uttar Pradesh, India.

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Abstract

Background: *This study aimed to characterize the AMR profiles and biochemical markers of pathogens from environmental sources to guide public health strategies.*

Materials and Methods: *From March 10 to April 10, 2025, a total of 340 environmental samples were collected from high-, moderate-, and low-risk areas in Pakbara, including soil (n=136), water (n=102), and surface swabs (n=102). Samples were cultured on selective media such as MacConkey and cetrimide agar. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method for amoxicillin, ciprofloxacin, vancomycin, and ceftriaxone, adhering to CLSI guidelines. Biochemical assays included nitrocefin tests for beta-lactamase and chrome azurol S (CAS) assays for siderophore production. Statistical analysis employed chi-square, ANOVA, and logistic regression, with significance set at $p<0.05$.*

Results: *Pathogenic organisms were detected in 30.6% (104/340) of samples, with water samples showing the highest prevalence (35.3%) and bacterial load (mean: 4.85 log CFU/mL). Escherichia coli (40.4%) and Staphylococcus aureus (29.8%) were the most frequently identified species. AMR prevalence ranged from 25.0% (vancomycin) to 32.7% (amoxicillin), and 17.3% of isolates were classified as multidrug-resistant. Beta-lactamase activity was present in 36.5% of isolates, and siderophore levels were notably elevated in water-derived samples. Both sample type ($\chi^2 = 12.45$, $p = 0.014$) and environmental risk level ($\chi^2 = 8.76$, $p = 0.013$) were significantly associated with AMR, while beta-lactamase production was a strong predictor of multidrug resistance (OR = 3.45, $p = 0.002$).*

Conclusion: *The study underscores the role of environmental water reservoirs in Pakbara as significant contributors to the spread of AMR pathogens. The high prevalence of beta-lactamase-producing and multidrug-resistant strains calls for immediate implementation of sanitation improvements and robust diagnostic surveillance in semi-urban regions to mitigate the public health risks posed by environmental AMR.*

Keywords: *antimicrobial resistance, environmental reservoirs, beta-lactamase, multidrug resistance, Pakbara, siderophores*

INTRODUCTION

Antimicrobial resistance (AMR) is a formidable global health crisis, contributing to approximately 1.27 million deaths annually and projected to cause 10 million deaths by 2050 if unchecked[1]. In low- and middle-income countries like India, environmental reservoirs—soil, water, and fomites—serve as critical conduits for resistant pathogens [2], [3], with AMR prevalence for beta-lactams (e.g., amoxicillin) ranging from 20–50% and fluoroquinolones (e.g., ciprofloxacin) from 15–30% in non-clinical settings [4]. Multidrug resistance (MDR), defined as resistance to three or more antibiotic classes, affects 10–25% of environmental isolates, driven by mobile genetic elements such as plasmids and integrons facilitating horizontal gene transfer (HGT) of resistance genes (e.g., blaTEM, qnrS) [5]. Biochemical signatures, including beta-lactamase production and siderophore synthesis, enhance resistance and virulence by hydrolyzing antibiotics and scavenging iron, respectively, posing challenges for therapeutic and diagnostic strategies [6]. Pakbara, a semi-urban Census Town in Moradabad district, India, was selected for its prototypical environmental profile, characterized by agricultural runoff rich in antibiotic residues, untreated wastewater discharge, and high-traffic public interfaces (e.g., marketplaces, ponds, bus stops) [7]. These conditions create ecological niches for pathogens like Escherichia coli [8], Staphylococcus aureus, Klebsiella pneumoniae, and Pseudomonas aeruginosa, promoting HGT and selective pressure [9]. Despite extensive AMR research, community-level studies in semi-urban India are limited, leaving gaps in understanding local resistance dynamics. Pakbara's proximity to industrial and agricultural activities, coupled with seasonal monsoon-driven water contamination, amplifies its relevance as a sentinel site for AMR surveillance. This study investigates AMR prevalence, MDR patterns, and biochemical signatures in Pakbara's environmental reservoirs to elucidate molecular and ecological drivers of resistance, aiming to inform targeted public health interventions, including enhanced sanitation, water treatment, and rapid diagnostic development for semi-urban settings.

MATERIALS AND METHODS

Study Design and Sample Collection

A cross-sectional study was conducted from March 10 to April 10, 2025, to characterize AMR and biochemical signatures of pathogens in environmental reservoirs in Pakbara, Moradabad, Uttar Pradesh, India. A total of 340 environmental samples were collected, stratified by type (136 soil, 40%; 102 water, 30%; 102 surface swabs, 30%) and risk level (136 high-risk, 40%; 102 moderate-risk, 30%; 102 low-risk, 30%). High-risk areas included marketplaces, moderate-risk areas included agricultural fields, and low-risk areas included remote sites. Soil samples (10–20 g) were collected from 0–5 cm depth using sterile spatulas and stored in sterile polyethylene bags at 4°C. Water samples (100–250 mL) from ponds, wells, and streams were collected in sterile polypropylene bottles, with sodium thiosulfate (0.1 mg/L) added for chlorinated sources to neutralize residual chlorine, and stored at 4°C. Surface swabs were taken from 10 cm^2 areas of public surfaces (e.g., bus stops, market countertops) using sterile cotton swabs moistened with 0.85% saline, placed in Stuart's transport medium, and stored at 4°C. All samples were processed within 24 hours to ensure microbial viability, following standard protocols (APHA, 2017).

Microbiological Analysis

Samples were analyzed to quantify and identify pathogens. Soil and water samples were serially diluted in sterile phosphate-buffered saline (PBS, pH 7.4), and swabs were vortexed in 1 mL PBS to release microbes. Aliquots (100 µL) were spread on selective media: blood agar for general pathogens, MacConkey agar for Gram-negative bacilli, and cetrimide agar for Pseudomonas aeruginosa. Plates were incubated aerobically at 37°C for 24–48 hours. Colony-forming units (CFU) were enumerated as log CFU/g (soil), log CFU/mL (water), or log CFU/cm^2 (swabs). Isolates were identified using Gram staining and biochemical tests, including catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate (IMViC), urease, and triple sugar iron (TSI) tests, following standard methods (Barrow and Feltham, 2003). Identified pathogens included Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, and P. aeruginosa. Quality control used American Type Culture Collection (ATCC) strains (E. coli ATCC 25922, S. aureus ATCC 25923).

Antimicrobial Susceptibility Testing

AMR was assessed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar, per Clinical and Laboratory Standards Institute (CLSI, 2025) guidelines. Tested antibiotics included amoxicillin (10 µg), ciprofloxacin (5 µg), vancomycin (30 µg), and ceftriaxone (30 µg). Bacterial suspensions were adjusted to 0.5

McFarland standard (approximately 1.5 × 10^8 CFU/mL) and inoculated onto agar plates. Antibiotic discs were applied, and plates were incubated at 37°C for 18–24 hours. Zone diameters (mm) were measured and interpreted as Sensitive, Intermediate, or Resistant per CLSI breakpoints. Multidrug resistance (MDR) was defined as resistance to three or more antibiotic classes. E. coli ATCC 25922 served as a positive control, and sterile discs as a negative control, ensuring assay reliability.

Biochemical Assays

Biochemical signatures were evaluated for resistance and virulence markers. Beta-lactamase production was detected using the nitrocefin assay, with a color change from yellow to red within 30 minutes indicating a positive result. Siderophore production was quantified using the chrome azurol S (CAS) liquid assay, with absorbance measured at 630 nm after 24-hour incubation at 37°C. Siderophore levels were categorized as High (>50% color change), Low (10–50%), or None (<10%), based on standard curves with deferoxamine. Assays were performed in triplicate, using P. aeruginosa ATCC 27853 as a positive control for siderophores (Schwyn and Neilands, 1987).

Statistical Analysis

Data were analyzed to evaluate pathogen prevalence, AMR patterns, and biochemical signatures. Descriptive statistics included frequencies and percentages for categorical variables (e.g., pathogen isolation, AMR status, MDR status) and means with standard deviations for continuous variables (e.g., pathogen load). Chi-square tests assessed associations between categorical variables, such as pathogen isolation versus sample type or AMR versus risk level. One-way ANOVA compared pathogen load across sample types and risk levels. Logistic regression identified predictors of MDR, including beta-lactamase production and risk level, with odds ratios (OR) and 95% confidence intervals. Analyses were conducted using Python libraries (pandas for data management, scipy for statistical tests, statsmodels for regression), with a significance threshold of $p<0.05$. All statistical models were validated by a statistician to ensure accuracy, and results were pooled across sample types for robust analysis. Data were formatted for compatibility with Microsoft Excel.

RESULT

Pathogen Prevalence across Sample Types and Risk Levels

A total of 340 environmental samples were collected from various sources including soil, water, and surface swabs across high, moderate, and low-risk zones. Out of these, 104 samples (30.6%) were positive for at least one bacterial pathogen (Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, or Pseudomonas aeruginosa). The highest prevalence was observed in soil samples from high-risk zones (44.4%), followed closely by water samples from high-risk zones (43.9%). In contrast, the lowest prevalence was observed in soil samples from low-risk zones (17.1%). Surface swabs showed a unique pattern with low-risk zones demonstrating slightly higher positivity (32.3%) compared to moderate (16.7%) and high-risk (24.4%) zones, indicating variable surface contamination patterns independent of zone classification as show in Table I.

Antimicrobial Resistance Patterns

Among the 104 pathogen-positive samples, antimicrobial resistance (AMR) was most pronounced in isolates from high-risk zones. Resistance to amoxicillin was most common, observed in 40.4% of high-risk isolates, followed by ceftriaxone (34.6%), ciprofloxacin (32.7%), and vancomycin (26.9%). Moderate and low-risk zones showed comparatively lower resistance rates, with amoxicillin resistance decreasing to 28.6% and 20.8% respectively. Overall, resistance patterns suggest a correlation between risk classification of the sampling site and the burden of antibiotic resistance as show in Table II.

Biochemical Signature in Pathogenic Isolates

Biochemical profiling of the 104 pathogen-positive isolates revealed that 36.5% (n=38) were beta-lactamase producers, signifying a substantial presence of resistance-conferring enzymes. Additionally, siderophore production was variable: 18.3% of isolates demonstrated high siderophore activity, 28.8% showed low activity, while 52.9% did not exhibit any detectable siderophore production. This indicates that a significant proportion of isolates possess virulence-associated traits that may enhance pathogenicity as show in Table III.

Pathogen Load by Sample Type and Risk Level

A total of 104 positive environmental samples were analyzed for pathogen load. Among the sample types, water samples exhibited the highest mean microbial load (4.85 log CFU/mL ± 0.80), followed by soil samples (4.52 log CFU/g ± 0.75) and surface swabs (4.38 log CFU/cm² ± 0.72). When analyzed by risk level, high-risk areas showed the highest mean pathogen load (4.79 ± 0.77 log CFU), compared to moderate-risk (4.55 ± 0.79 log CFU) and low-risk areas (4.41 ± 0.74 log CFU). These findings indicate that both sample type and environmental risk level significantly influence microbial contamination, with water sources and high-risk areas representing the most critical reservoirs as show in Table IV.

Chi-Square Tests

Chi-square analyses revealed significant associations between sample type and E. coli isolation ($\chi^2 = 12.45$, $p = 0.014$), with E. coli more frequently isolated from water samples. Additionally, a significant association was observed between risk level and amoxicillin resistance ($\chi^2 = 8.76$, $p = 0.013$), indicating a higher resistance rate in samples from high-risk areas as show in Table V.

ANOVA Tests

One-way ANOVA showed that pathogen load significantly varied by sample type ($F = 3.21$, $p = 0.043$) and risk level ($F = 4.87$, $p = 0.009$). These findings suggest environmental and risk-based factors influence microbial burden as show in Table VI.

Logistic Regression: Predictors of Multidrug Resistance

Binary logistic regression identified beta-lactamase production as a strong predictor of multidrug resistance (OR = 3.45, p = 0.002). High-risk environmental sites also significantly predicted MDR occurrence (OR = 2.12, p = 0.045), highlighting their role in antimicrobial resistance dissemination as show in Table VII.

DISCUSSION:

The finding of 30.6% pathogen positivity (104/340 samples) in Pakbara's environmental reservoirs closely aligns with environmental surveillance studies in South Asia, which report 20–40% bacterial contamination in non-clinical soil, water, and fomites **(Walsh et al., 2011; Purohit et al., 2017)** [10], [11]. The predominance of Escherichia coli (40.4% of isolates) and Staphylococcus aureus (29.8%) reflects their ecological resilience and frequent dissemination through human-animal-environment interfaces, consistent with **Laxminarayan et al., (2013)** [12], who identified these species as prevalent in Indian community settings due to their ability to survive diverse environmental stressors. Water samples demonstrated the highest pathogen prevalence (35.3%, 36/102) and load (mean 4.85 log CFU/mL), corroborated by **Brower et al., (2020)** [13]**,** who reported elevated E. coli and Klebsiella pneumoniae in Indian water bodies contaminated by fecal matter, agricultural runoff, and untreated wastewater. The significant association between sample type and pathogen isolation (chi^2 = 12.45, p = 0.014) underscores water's role as a primary AMR reservoir, likely exacerbated by Pakbara's monsoon-influenced hydrology and inadequate sanitation infrastructure, as noted in regional studies **(Purohit et al., 2017)** [10]. Soil samples (31.6% positivity) and surface swabs (24.5%) exhibited lower prevalence, possibly due to reduced microbial persistence in terrestrial environments compared to aquatic systems, aligning with **Zhang et al., (2018)** [14]**,** who found water to be a more stable niche for microbial proliferation.

AMR rates, ranging from 25.0% (vancomycin) to 32.7% (amoxicillin) across 104 positive isolates, are consistent with Indian environmental data reporting 20–50% resistance to beta-lactams and 15–30% to fluoroquinolones **(Gandra et al., 2017)** [15]**.** The elevated amoxicillin resistance (40.4% in high-risk areas such as marketplaces and ponds) mirrors global trends driven by widespread dissemination of bla TEM and bla CTX-M genes encoding extended-spectrum beta-lactamases (ESBLs) **(Antimicrobial Resistance Collaborators 2022)** [16]. Ceftriaxone resistance (30.8%) and ciprofloxacin resistance (28.8%) align with **Brower et al., (2017)** [13] who documented 25–40% resistance to third-generation cephalosporins and fluoroquinolones in Indian wastewater, attributed to selective pressures from antibiotic residues and heavy metals. The 17.3% MDR prevalence (18/104 isolates) falls within the 10–25% range reported for environmental MDR in India, often mediated by mobile genetic elements such as plasmids, integrons, and transposons carrying resistance genes like qnr S (fluoroquinolone resistance) and aac (6')-Ib-cr (aminoglycoside resistance) **(Laxminarayan et al., 2013)** [12]**.** The significant association between AMR and risk level (chi^2 = 8.76, p = 0.013 \)) highlights high-traffic areas as hotspots for horizontal gene transfer, consistent with

Walsh et al. (2011) [11]**,** who identified urban interfaces as amplifiers of AMR due to anthropogenic activities, including improper waste disposal and antibiotic overuse.

Biochemical signatures offer critical insights into resistance and virulence mechanisms. Beta-lactamase production in 36.5% of isolates (38/104), predominantly among amoxicillin-resistant strains, aligns with **Purohit et al., (2017)** [10], who reported 30–50% beta-lactamase positivity in Indian environmental E. coli and K. pneumoniae. The logistic regression result linking beta-lactamase to MDR (OR = 3.45, p = 0.002) supports molecular studies identifying ESBL genes as co-resistance markers, often co-located with aminoglycoside and fluoroquinolone resistance genes on multidrug plasmids **(Brower et al., 2017)** [13]. Siderophore production, with 18.3% high and 28.8% low levels, was notably elevated in water samples, consistent with **Zhang et al., (2018)** [14] who linked siderophores to enhanced microbial fitness in iron-limited aquatic environments. Siderophores facilitate iron scavenging, promoting biofilm formation and virulence, which may explain the higher pathogen load in water (ANOVA (F = 3.21, p = 0.043). The elevated load in high-risk areas (mean 4.79 log CFU, ANOVA (F = 4.87, p = 0.009) corroborates **Antimicrobial Resistance Collaborators (2022)** [16]**,** who associated anthropogenic activity with increased microbial proliferation and resistance gene dissemination.

The observed vancomycin resistance (25.0%) was higher than anticipated compared to **Gandra et al. (2017)** [15]**,** who reported <15% in Indian S. aureus. This discrepancy may stem from local antibiotic misuse, cross-contamination from healthcare facilities, or dissemination of vanA/vanB genes, as reported in environmental Enterococcus species **(Walsh et al., 2011)** [11]. Similarly, the 25–30% resistance to ciprofloxacin and ceftriaxone suggests broader dissemination of plasmid-mediated resistance genes, potentially linked to agricultural and industrial runoff in Pakbara, as noted by **Purohit et al. (2017)** [10]. These findings position Pakbara as a sentinel site for AMR surveillance, given its environmental profile—characterized by antibiotic-laden runoff, untreated wastewater, and high-traffic public areas—which mirrors conditions in other semi-urban Indian settings **(Laxminarayan et al., 2013)** [12]. The ecological drivers in Pakbara, including monsoon-driven water contamination and proximity to industrial activities, enhance its relevance for studying community-level AMR dynamics.

CONCLUSION:

This study demonstrates that environmental reservoirs in Pakbara, particularly water and high-risk public areas, are significant drivers of antimicrobial resistance (AMR), with 30.6% pathogen positivity, 25.0–32.7% resistance rates, and 17.3% multidrug resistance (MDR). The high prevalence of beta-lactamase (36.5%) and siderophores in water samples highlights the role of biochemical signatures in resistance and virulence, necessitating targeted public health interventions. These findings underscore the urgent need for improved sanitation, wastewater treatment, and antibiotic stewardship to mitigate AMR spread in semi-urban India, aligning with the World Health Organization's Global Action Plan on AMR (WHO, 2015) [17]. The development of rapid diagnostic assays targeting beta-lactamase and siderophores is critical to enhance local laboratory capacity and guide treatment strategies.

Limitations: The study's cross-sectional design limits temporal insights into resistance trends, and the absence of whole-genome sequencing restricts identification of specific resistance genes (e.g., blaNDM-1, qnrS). The nitrocefin assay detects broad beta-lactamase activity but does not differentiate ESBLs from carbapenemases, potentially underestimating complex resistance profiles. Lack of source attribution (e.g., human vs. agricultural inputs) constrains causal inference.

Future Prospects: Future research should employ metagenomic sequencing to characterize resistome dynamics, longitudinal sampling to track seasonal AMR variations, and source-tracking studies to quantify anthropogenic contributions. Integrating these approaches will strengthen AMR surveillance and inform evidence-based interventions in semi-urban settings.

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Table I: Pathogen Prevalence by Sample Type and Risk Level

Sample Type	Risk Level	Total Samples	Positive Samples	Prevalence (%)
Soil	High	54	24	44.4
Soil	Moderate	41	12	29.3
Soil	Low	41	7	17.1
Water	High	41	18	43.9
Water	Moderate	31	11	35.5
Water	Low	30	7	23.3
Surface Swab	High	41	10	24.4
Surface Swab	Moderate	30	5	16.7
Surface Swab	Low	31	10	32.3

Prevalence calculated as (Positive Samples / Total Samples) × 100. Pathogens include E. coli, S. aureus, K. pneumoniae, P. aeruginosa.

Table II: AMR Rates by Antibiotic and Risk Level

Antibiotic	Risk Level	Positive Samples	Resistant Samples	Resistance Rate (%)

Amoxicillin	High	52	21	40.4
Amoxicillin	Moderate	28	8	28.6
Amoxicillin	Low	24	5	20.8
Ciprofloxacin	High	52	17	32.7
Ciprofloxacin	Moderate	28	8	28.6
Ciprofloxacin	Low	24	5	20.8
Vancomycin	High	52	14	26.9
Vancomycin	Moderate	28	7	25.0
Vancomycin	Low	24	5	20.8
Ceftriaxone	High	52	18	34.6
Ceftriaxone	Moderate	28	8	28.6
Ceftriaxone	Low	24	6	25.0

Resistance Rate = (Resistant Samples / Positive Samples) * 100. Based on 104 positive isolates.

Table III: Biochemical Signatures in Positive Isolates

Biochemical Marker	Frequency (n)	Percentage (%)
Beta-Lactamase Positive	38	36.5
Siderophore High	19	18.3
Siderophore Low	30	28.8
Siderophore None	55	52.9
Total	104	100.0

Percentages based on 104 positive isolates. Beta_Lactamase via nitrocefin assay; Siderophore Production via CAS assay.

Table IV: Pathogen Load by Sample Type and Risk Level

Group	Subgroup	Positive Samples (n)	Load (log CFU)
Sample Type	Soil	43	4.52 ± 0.75
	Water	36	4.85 ± 0.80
	Surface Swab	25	4.38 ± 0.72
Risk Level	High	52	4.79 ± 0.77
	Moderate	28	4.55 ± 0.79
	Low	24	4.41 ± 0.74

Load expressed in log CFU per gram (soil), per mL (water), or per cm^2 (surface swabs). Based on 104 positive isolates.

Table V Chi-Square Analysis of Environmental and Microbial Characteristics

Variable Pair	χ^2 Value	p-value
Sample type vs. E. coli isolation	12.45	0.014
Risk level vs. Amoxicillin resistance	8.76	0.013

Chi-square test was used to assess associations between categorical variables. A p-value < 0.05 was considered statistically significant.

Table VI. One-Way ANOVA of Pathogen Load by Sample Type and Risk Level

Factor	F-value	p-value
Sample Type	3.21	0.043
Risk Level	4.87	0.009

One-way ANOVA was conducted to compare mean pathogen load (log CFU) among sample types and risk levels. A p-value < 0.05 indicated statistical significance.

Table VII. Logistic Regression: Predictors of Multidrug Resistance (MDR)

Predictor Variable	Odds Ratio (OR)	p-value
Beta-lactamase Production	3.45	0.002
High-Risk Environmental Site	2.12	0.045