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Research Article

Occurrence of Multidrug-Resistant *Escherichia Coli* and *Klebsiella Pneumoniae* and Associated Virulence Genes in Environmental Samples from Ekiti State, Southwestern Nigeria

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Abstract

Background: Multidrug-Resistant (MDR) Enterobacteriaceae are an emerging public health threat, with environmental reservoirs facilitating their persistence and dissemination. This study investigated the occurrence, antimicrobial resistance profiles, and virulence gene carriage of *Escherichia coli* and *Klebsiella pneumoniae* in environmental samples from Ado-Ekiti, Ekiti State, Nigeria.

Methods: A cross-sectional descriptive study was conducted on one hundred environmental samples, including soil from dumpsites and farmlands, and sewage swabs. Isolation of Enterobacteriaceae was performed using MacConkey and Eosin Methylene Blue agars, followed by biochemical identification. Antimicrobial susceptibility testing against twelve antibiotics was carried out using the Kirby–Bauer disc diffusion method according to CLSI (2021) guidelines. Phenotypic carbapenem resistance was assessed using Brilliance™ ESBL Agar and Brilliance™ CRE Agar. Virulence-associated genes (*fimH, rmpA, eae, hlyA*) were detected by polymerase chain reaction.

Results: Of the 50 Enterobacteriaceae isolates recovered, 25 (68%) were *E. coli* and 12 (32%) were K. pneumoniae. High levels of multidrug resistance were observed across all isolates. Dumpsite isolates demonstrated 100% resistance to ampicillin and gentamicin; sewage isolates were 100% resistant to amoxicillin–clavulanate, ampicillin, and gentamicin; and farmland isolates showed 100% resistance to ampicillin and gentamicin. MDR phenotypes, defined as resistance to more than 3 antibiotic classes, were observed in 76% of *E. coli* and 50% of *K. pneumoniae* isolates. Carbapenem resistance was absent in dumpsite isolates but detected in 6% and 1% of sewage- and farmland-derived isolates, respectively. Molecular analysis revealed the presence of *fimH* and *rmpA* in *K. pneumoniae* and *eae* in *E. coli*, indicating potential virulence.

Conclusion: Dunghills, sewage, and farmlands in Ado-Ekiti harbour multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* carrying virulence genes. These findings highlight environmental reservoirs as critical sources of antimicrobial resistance, emphasizing the need for surveillance and improved waste management to curb the spread of resistant pathogens.

Keywords: *Escherichia coli, Klebsiella pneumoniae*, Multidrug resistance, Virulence genes, Environmental isolates

Introduction

Antimicrobial Resistance (AMR) has emerged as one of the most pressing public health threats of the 21st century. The World Health Organization (WHO) identifies AMR as a major risk to human, animal, and environmental health, warning that drug-resistant infections could result in as many as 10 million deaths annually by 2050 if effective control measures are not implemented [1]. This challenge is particularly severe in low- and middle-income countries, where inappropriate antibiotic use, limited access to diagnostic services, and inadequate waste management systems contribute significantly to the emergence and spread of resistant microorganisms.

Within this context, members of the Enterobacteriaceae family, especially *Escherichia coli* and *Klebsiella pneumoniae,* play a central role in AMR-related infections. These organisms are responsible for a wide range of community-acquired and

healthcare-associated infections and have increasingly exhibited resistance to multiple classes of antibiotics [2]. Of particular concern is resistance to carbapenems, which are often reserved as last-line agents for severe gram-negative infections. Carbapenem-resistant Enterobacteriaceae are classified as urgent public health threats by both the Centers for Disease Control and Prevention (CDC) and the WHO [3]. Resistance in these organisms is frequently mediated by plasmid-encoded carbapenemases, including *Klebsiella Pneumoniae* Carbapenemase (KPC), New Delhi Metallo-β-lactamase (NDM), Verona Integron-encoded Metallo-β-lactamase (VIM), and oxacillinase-48 (OXA-48), all of which facilitate rapid horizontal gene transfer within and across bacterial species [4].

Although carbapenem-resistant Enterobacteriaceae were initially regarded as predominantly hospital-associated pathogens, accumulating evidence now highlights the importance of environmental reservoirs in sustaining and disseminating resistant strains. Agricultural soils, wastewater, surface water, and animal faeces serve as important niches for multidrug-resistant bacteria and antimicrobial resistance genes [5,6]. These pathways are especially relevant in regions where antibiotics are widely used in animal husbandry and where untreated or poorly treated waste is discharged into the environment.

In Nigeria, including Ekiti State, challenges such as inadequate sewage infrastructure, indiscriminate disposal of animal manure, and close human–animal interactions create favorable conditions for the persistence and spread of multidrug-resistant bacteria in the environment. Despite these risks, routine environmental surveillance for resistant Enterobacteriaceae remains limited.

Against this background, the present study investigated the occurrence of multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae,* along with their associated virulence genes, in environmental samples obtained from dunghills, sewage discharge points, and farmlands in and around Ado-Ekiti, Ekiti State, Southwestern Nigeria. Understanding the prevalence, resistance patterns, and virulence profiles of these organisms in environmental reservoirs is essential for informing effective strategies aimed at mitigating antimicrobial resistance and safeguarding public health.

Materials And Methods

Sample Collection

A total of 100 surface soil samples were collected from selected dunghills and farmlands within Ado-Ekiti. Additionally, sewage swab samples were collected from designated sewage discharge sites. All samples were collected aseptically using sterile universal containers, properly labelled, and transported promptly to the Microbiology Laboratory of Ekiti State University, Ado-Ekiti, for microbiological analysis.

Isolation of Enterobacteriaceae from Soil Samples

Soil samples were collected aseptically using sterile spatulas and transferred into sterile, labelled polyethene containers. Samples were transported to the laboratory at ambient temperature and processed within 24 hours to minimize changes in microbial composition.

Ten grams of each soil sample were aseptically weighed and suspended in 90mL of sterile physiological saline (0.85% NaCl) to obtain a 10^{-1} dilution. The suspension was homogenized by vigorous shaking for 2 to 3 minutes, followed by preparation of serial ten-fold dilutions ranging from 10^{-2} to 10^{-6}.

Aliquots of 0.1 mL from appropriate dilutions were inoculated onto MacConkey agar and Eosin Methylene Blue (EMB) agar plates using the spread-plate technique. Plates were incubated aerobically at 37°C for 18 to 24 hours. After incubation, colonies showing typical Enterobacteriaceae characteristics, including lactose fermentation on MacConkey agar and green metallic sheen or characteristic coloration on EMB agar, were enumerated and selected. Distinct colonies were repeatedly subcultured on nutrient agar to obtain pure isolates, which were subsequently preserved on nutrient agar slants at 4°C for further analyses.

Isolation of Enterobacteriaceae from Sewage Swab Samples

Sewage samples were collected aseptically using sterile cotton swabs by swabbing the inner surfaces of sewage channels at selected locations. Each swab was immediately transferred into a sterile test tube containing 10mL of sterile physiological saline and transported to the laboratory for immediate processing. The swab-containing tubes were vortexed vigorously for 1 to 2 minutes to release microbial cells into suspension. Serial dilutions (10^{-1} to 10^{-6}) were prepared, and aliquots of 0.1 mL were inoculated onto MacConkey agar and EMB agar plates.

The inoculated plates were incubated aerobically at 37°C for 18 to 24 hours. Following incubation, lactose-fermenting and non-lactose-fermenting colonies on MacConkey agar and characteristic colonies on EMB agar were identified. Presumptive *E. coli* isolates were recognized by metallic green sheen colonies, while mucoid pink to purple colonies were suggestive of Klebsiella species. Representative colonies were subcultured repeatedly to obtain pure cultures, which were stored on nutrient agar slants at 4°C.

Presumptive and Confirmatory Identification

Presumptive identification of Enterobacteriaceae was based on Gram-negative rod morphology, lactose fermentation on MacConkey agar, and oxidase negativity. Confirmatory identification was carried out using standard biochemical tests, including Triple Sugar Iron agar, citrate utilization, urease, indole, and motility tests, following established microbiological protocols.

Quality Control

Quality assurance was ensured by processing reference strains *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13883 alongside test isolates to validate culture media and biochemical reactions.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using the disk diffusion method on Mueller–Hinton agar. The antibiotics tested included cefotaxime, cotrimoxazole, gentamicin, cefuroxime, chloramphenicol, ceftriaxone, amikacin, vancomycin,

chlorpromazine, tetracycline, ciprofloxacin, and meropenem. Plates were incubated at 37 °C for 18 hours, after which zones of inhibition were measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [7].

Phenotypic Detection of Carbapenem Resistance

Phenotypic screening for carbapenem resistance was conducted using imipenem (10 µg) and meropenem (10 µg) disks, following CLSI recommendations [7]. Standardized bacterial suspensions equivalent to 0.5 McFarland turbidity were inoculated onto Mueller–Hinton agar plates. Antibiotic disks were applied, and plates were incubated at 37°C for 24 hours. Zone diameters were measured and interpreted accordingly.

Brilliance™ ESBL and Brilliance™ CRE Agar Screening

Presumptive resistant isolates were further screened on Brilliance™ ESBL Agar and Brilliance™ CRE Agar following the manufacturer's instructions. Plates were inoculated directly with bacterial colonies and incubated aerobically at 37°C for 18 to 24 hours. Presumptive ESBL- and CRE-producing isolates were identified based on characteristic colour development.

Molecular Detection of Virulence Genes

Molecular detection of selected virulence genes was carried out using conventional polymerase chain reaction (PCR). Genomic DNA was extracted from selected isolates, and amplification was performed using gene-specific primers previously described in the literature. Each PCR reaction contained SYBR Green buffer, $MgCl_2$, dNTPs, forward and reverse primers, Taq DNA polymerase, and sterile distilled water, with template DNA added to a final volume of 12.5µL. Negative controls were included to rule out false amplification. PCR amplification was conducted using a GeneAmp 9700 thermal cycler, and amplified products were visualized by agarose gel electrophoresis (Table 1).

Table 1: List of primers and amplification conditions.

Gene	Primer orientation	Primer sequence	Fragment size	Profile
Klebsiella pneumonia				
RmpA	forward	CATAAGAGTATTGGTTGACAG	461	5 min at 94°C, followed by 35 cycles of 10 s at 95°C, 30 s at 52°C, and 40 s at 72°C, and the final elongation of 3 min at 72°
	Reverse	CTTGCATGAGCCATCTTTCA		
FimH	forward	TGCTGCTGGGCTGGTCGATG	506	5 min at 94°C, followed by 35 cycles of 10 s at 95°C, 30 s at 50°C, and 30 s at 72°C, and the final elongation of 3 min at 72°
	Reverse	GGGAGGGTGACGGTGACATC		
Escherichia coli				
Eae	forward	TCA ATG CAG TTC CGT TAT CAG TT	482	5 min at 94°C, followed by 35 cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and the final elongation of 3 min at 72°
	Reverse	G T A A A G T C C G T - TACCCCAACCTG		
HlyA	forward	AGCTGCAAGTGCGGGTCTG	569	5 min at 94°C, followed by 35 cycles of 10 s at 95°C, 30 s at 48°C, and 30 s at 72°C, and the final elongation of 3 min at 72°
	Reverse	TACGGGTTATGCCTGCAAGT-TCAC		
	Reverse	TTGACTTCCTCCAGGCTG 50.3	1675	

Results

Table 2: Percentage Frequency Distribution of Environmental Enterobacteriaceae Isolates.

Source	Enterobacteriaceae (n)	*E. coli* n (%)	*Klebsiella spp.* n (%)
Dunghill	7	6 (85.7%)	1 (14.3%)
Sewage	18	13 (72.2%)	5 (27.8%)
Farmland	12	6 (50.0%)	6 (50.0%)
Total	37	25 (68.0%)	12 (32.0%)

A total of 87 bacterial isolates were obtained from environmental samples, of which 50 belonged to the Enterobacteriaceae family. Based on cultural characteristics and biochemical identification, 37 isolates were confirmed as *Escherichia coli* and *Klebsiella pneumoniae*. Sewage samples yielded the highest number of Enterobacteriaceae isolates, followed by farmland and dunghill samples. Overall, *E. coli* was the dominant organism, accounting for more than two-thirds of the identified isolates, while *Klebsiella species* constituted about one-third. The distribution of *E. coli* and Klebsiella across the different sampling sites is presented in Table 2.

Antibiotic susceptibility testing revealed marked differences in resistance patterns between organisms and across antibiotic classes. All *E. coli* isolates showed complete resistance to ampicillin, while resistance to cefotaxime was comparatively lower. Similarly, *K. pneumoniae* isolates were uniformly resistant to ampicillin and

nitrofurantoin, but showed the least resistance to levofloxacin. A high burden of multidrug resistance was observed, with over three-quarters of E. coli isolates and half of the K. pneumoniae isolates exhibiting resistance to three or more classes of antibiotics, as illustrated in Figure 1.

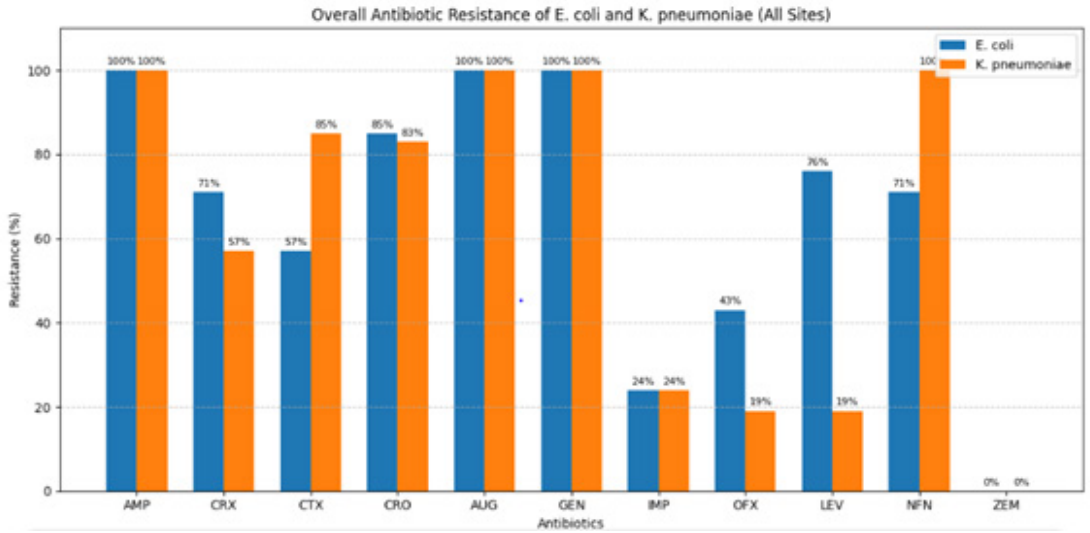

Figure 1: Antibiotic resistance profiles of *Escherichia coli* and *Klebsiella pneumoniae* isolates from selected study sites.

Phenotypic screening of multidrug-resistant isolates for carbapenem resistance showed notable variation by sample source. No carbapenem resistance was detected among isolates recovered from dumpsite samples. In contrast, sewage samples accounted for the highest proportion of carbapenem-resistant *E. coli,* while all sewage-derived *K. pneumoniae* remained susceptible. Farmland samples also yielded carbapenem-resistant isolates from both organisms, although at lower frequencies. Overall, approximately one-quarter of the multidrug-resistant isolates demonstrated phenotypic carbapenem resistance, with detailed distribution shown in Table 3 and isolation patterns illustrated in Figure 2.

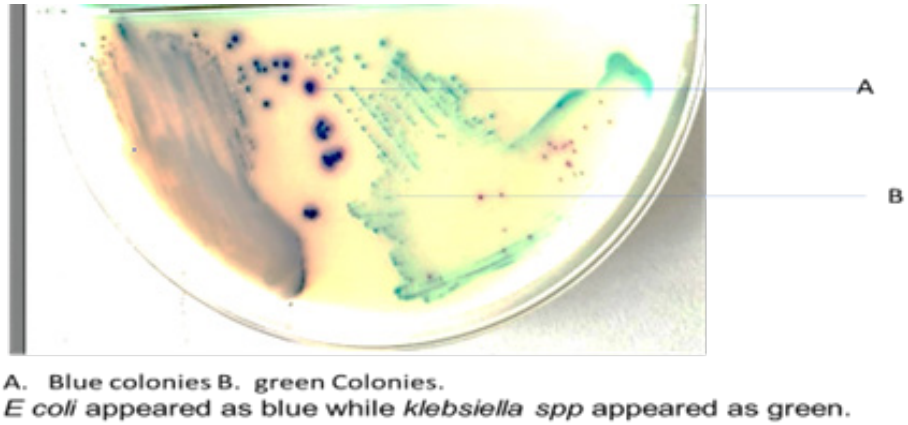

Figure 2: Isolation of Carbapenem-Resistant Enterobacteriaceae on Brilliance™ ESBL and Brilliance™ CRE Agar.

Table 3: Distribution of Phenotypic Carbapenem Resistance among Multidrug-Resistant (MDR) Isolates from Environmental Samples.

Sample Source	Organism	MDR Isolates Tested (n)	Carbapenem-Resistant n (%)	Carbapenem-Susceptible n (%)
Dumpsite	***Escherichia coli***	7	0 (0.0)	7 (100)
	Klebsiella pneumoniae	2	0 (0.0)	2 (100)
Subtotal	—	**9**	**0 (0.0)**	**9 (100)**
Sewage	***Escherichia coli***	11	4 (36.4)	7 (63.6)
	Klebsiella pneumoniae	0	0 (0.0)	0 (0.0)
Subtotal	—	**11**	**4 (36.4)**	**7 (63.6)**

Farmland	***Escherichia coli***	6	1 (16.7)	5 (83.3)
	Klebsiella pneumoniae	2	1 (50.0)	1 (50.0)
Subtotal	—	**8**	**2 (25.0)**	**6 (75.0)**
Grand Total	—	25	6 (24.0)	19 (76.0)

Molecular analysis of selected multidrug-resistant isolates confirmed the presence of important virulence-associated genes. Among *K. pneumoniae* isolates, the adhesion-related gene *fim*H was frequently detected, whereas the hypervirulence-associated gene *rmp*A was found in only one isolate. In *E. coli,* the *eae* gene was detected in all tested isolates, indicating a strong association with intestinal pathogenicity, while the *hly*A gene was absent. Representative PCR amplification results for these virulence genes are shown in Figure 3.

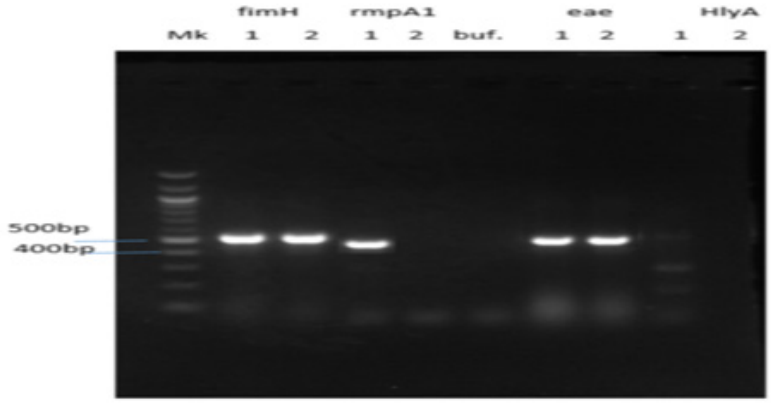

Figure 3: Agarose gel electrophoresis of PCR-amplified virulence genes from selected *K. pneumoniae* and E. coli isolates. Lane labels correspond to individual isolates. Bands were detected for *fim*H (~506 bp) and *rmp*A (~461 bp) in *K. pneumoniae*, and for eae (~482 bp) in *E. coli*. The *hly*A gene (~569 bp) was not detected in any *E. coli* isolate.

Footnote: Carbapenem resistance was determined phenotypically using disk diffusion and Brilliance™ ESBL Agar/ Brilliance™ CRE Agar in accordance with CLSI (2021) guidelines. Percentages were calculated based on the number of MDR isolates per organism at each sample source.

Discussion

The findings of this study reveal a substantial burden of multidrug-resistant Enterobacteriaceae in environmental sources, including dunghills, sewage, and farmlands. *Escherichia coli* emerged as the predominant organism, followed by *Klebsiella pneumoniae,* consistent with reports highlighting their widespread distribution and adaptability in environmental settings [8,9]. Sewage and agricultural environments, in particular, are increasingly recognized as critical reservoirs for resistant bacteria and resistance genes, especially in regions with limited wastewater treatment infrastructure and unregulated antibiotic use [10,11].

The high levels of resistance observed, including universal resistance to ampicillin, reflect the declining efficacy of commonly used antibiotics against gram-negative bacteria [11,12]. Such resistance patterns are likely driven by selective pressure from antibiotic residues in environmental matrices originating from human and animal sources. The high prevalence of multidrug resistance underscores the public health significance of environmental reservoirs as sources of transferable resistance determinants.

Although carbapenem resistance was less frequent, its detection in environmental isolates is alarming. Carbapenem-resistant Enterobacteriaceae are classified as critical priority pathogens due to limited therapeutic options and associated high mortality [12]. The presence of carbapenem resistance in sewage and farmland samples suggests ongoing dissemination of carbapenemase genes within environmental microbial communities.

The detection of virulence genes such as fimH, rmpA, and eae further highlights the pathogenic potential of environmental isolates. These genes enhance bacterial adhesion, persistence, and pathogenicity, indicating that environmental strains may pose risks comparable to clinical isolates [10,11].

Conclusion

Dunghills, sewage systems, and farmlands in Ado-Ekiti serve as important reservoirs of multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* carrying clinically relevant virulence genes. These findings underscore the critical role of environmental compartments in the epidemiology of antimicrobial resistance and emphasize the urgent need for strengthened environmental surveillance, improved waste management practices, and effective antibiotic stewardship to reduce the spread of resistant pathogens.

Acknowledgement

None.

Conflict of Interest

None.

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