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Colistin Resistance in WHO-Designated Global Priority Pathogens Isolated from Wastewater Effluents of Two Hospitals in Enugu Metropolis, South East Nigeria

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ABSTRACT

Introduction: Hospitals are breeding grounds for multidrug-resistant (MDR) bacteria, posing treatment challenges and increasing the risk of spreading "superbugs." This study investigates the prevalence of colistin-resistant bacteria, a last-resort antibiotic, in wastewater from tertiary hospitals in Enugu, Nigeria. **Methods**: Twenty wastewater samples were collected over three months from two tertiary hospitals in Enugu. A standardized protocol by the American Public Health Association (APHA) was followed. Samples were collected aseptically from key drainage points and transported to the lab within 2 hours. Bacteria were isolated using the pour-plate method and characterized by morphological and biochemical tests, including Catalase, Oxidase, and Glucose Fermentation. Antibiotic susceptibility was assessed using Kirby-Bauer disc diffusion, and colistin resistance was confirmed via broth microdilution. Multiplex PCR detected *mcr* genes indicating plasmid-mediated resistance. Data were analyzed using SPSS version 23 with Chi-Square and ANOVA tests at a significance level of $P < 0.05$. **Results**: Gram-negative bacteria were isolated from 63.1% of samples, with *Klebsiella* spp. being the most prevalent, accounting for 24.6%. Colistin resistance was phenotypically observed in *E. coli* (83%), *Klebsiella* spp. (75%), and *Pseudomonas aeruginosa* (100%). Genotypically, *E. coli* harbored *mcr-1* (17%) and *mcr-3* (83%), while all *Klebsiella* and *Pseudomonas* isolates carried multiple *mcr* genes. Additionally, these bacteria showed resistance to multiple antibiotics, including Septrin, Gentamycin, and Ceftriaxone. **Conclusion**: The significant presence of colistin-resistant bacteria, especially *E. coli* and *Klebsiella*, poses a public health concern, potentially leading to treatment failures and spreading resistance genes. Stricter monitoring of hospital wastewater is necessary to identify emerging resistance trends and improve antibiotic practices in hospitals.

INTRODUCTION

Antimicrobial resistance (AMR) is a growing public health crisis, threatening the effectiveness of antibiotics used to treat common infections. This is particularly concerning for last-resort antibiotics like colistin, which are crucial for treating multidrug-resistant bacteria. The emergence and spread of colistin resistance in environmental reservoirs like hospital wastewater is a significant public health concern [1]. Hospital wastewater represents a significant source of antimicrobial-resistant bacteria and resistance genes, which can potentially spread to the environment and human populations [2]. For example, a study by Kamaruzzaman *et al*. (2019) [3] demonstrated the presence of diverse antimicrobial-resistant bacteria in hospital wastewater, highlighting the potential for environmental dissemination. In 2019, Murray *et al*. (2022) [4] highlighted that approximately 50,000 deaths reported in sub-Saharan Africa were attributed to bacterial antimicrobial resistance, particularly in infections such as sepsis, pneumonia, and urinary tract infections. The rising death rates due to multidrug-resistant bacterial infections, including those resistant to colistin, underscore the growing concern that we may soon face a period where antibiotics are ineffective [4].

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Bacteria such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species have been designated by the World Health Organization (WHO) as global priority pathogens [5]. These bacteria are frequently associated with severe healthcare-acquired infections (HAIs) in hospital settings and often exhibit limited or no antimicrobial treatment options due to AMR [5]. These bacteria, along with other drug-resistant bacteria, can be passed from infected and colonized hospital patients through their excreta, along with antimicrobial residues, making hospital wastewater an ideal medium for the transfer of resistance genes between clinical and environmental bacteria [2, 6]. Research investigating the efficacy of wastewater treatment facilities indicates that neither primary nor secondary treatment methods can effectively eradicate multidrug-resistant bacteria from sewage [7]. Consequently, these resilient bacteria and their genes, which survive the treatment processes in hospital sewage, have the capacity to disseminate and persist in the environment. This presents a continuous source of antimicrobial resistance (AMR) and an ongoing health hazard for both humans and animals [8].

To guide the appropriate use of antibiotics and combat antimicrobial resistance, the World Health Organization (WHO) categorizes antibiotics into three groups based on their importance in human medicine and the risk of resistance development [9]. The "Access" group includes antibiotics that are generally effective against common infections and have a lower risk of resistance emergence, such as sulfamethoxazole/trimethoprim, many penicillins, tetracyclines, clindamycin, and amikacin [10]. The "Watch" group comprises antibiotics that are critically important for human medicine but have a higher risk of selecting for resistant strains. This group includes third-generation cephalosporins like ceftazidime and cefotaxime, piperacillin/tazobactam, ciprofloxacin, carbapenems, and vancomycin [10]. These antibiotics are also designated as "Essential Antimicrobial Agents for Human Medical Use" [11]. Finally, the "Reserve" group includes antibiotics considered "last-resort" options, reserved for treating infections caused by multidrug-resistant organisms, particularly those producing carbapenemases. Colistin, ceftazidime/avibactam, ceftolozane/tazobactam, intravenous fosfomycin, tigecycline, and linezolid belong to this group [10]. Preserving the effectiveness of "Reserve" antibiotics is crucial, and their use should be carefully controlled and monitored through robust stewardship programs**.**

This study focuses on colistin, a "Reserve" antibiotic, and investigates its presence in hospital wastewater in Enugu, Nigeria. The study specifically targets WHO-designated priority pathogens, known for their potential to cause severe infections and spread resistance. Identifying colistin resistance in these pathogens within hospital wastewater is critical for informing public health interventions and strengthening antibiotic stewardship efforts**.**

Colistin is a polypeptide antibiotic that belongs to the polymyxin family and is commonly known as polymyxin E [12]. It is an old antibiotic that is increasingly being used as a last-resort treatment for multidrug-resistant Gram-negative infections [13, 14], and it functions by electrostatically binding to the negatively charged lipid A component of bacterial lipopolysaccharide (LPS) [15]. Resistance to colistin can arise from modifications or the total absence of lipid A, leading to a decrease or elimination of the negative charge in lipopolysaccharide molecules. This alteration diminishes the electrostatic attraction between colistin and the bacterial cell [12]. Resistance can also occur due to the synthesis of enzymes that inactivate or modify the antibiotic, such as phosphotransferases that modify lipid A [16]; alterations in the bacterial cell membrane that hinder antibiotic uptake and target modification that prevents interaction with the antibiotic [17]; development of metabolic pathways that bypass the antibiotic's site of action and overexpression of efflux pumps that actively remove the antibiotic from the bacterial cell [18].

This study focuses on two large hospitals in Enugu, Nigeria: The University of Nigeria Teaching Hospital (UNTH) and the National Orthopedic Hospital Enugu (NOHE). These institutions were selected because they serve a substantial and densely populated region and manage a wide array of patients, including those with complex infections, which may contribute to a higher prevalence of antimicrobial-resistant (AMR) bacteria. Furthermore, Enugu, in general**,** and the hospitals included in this study, face challenges with sanitation infrastructure and waste management, further exacerbating the spread of resistant bacteria. For example, Oli *et al*. (2019) [19] reported that Enugu's inadequate sanitation infrastructure and improper waste management practices create environments conducive to the emergence and transmission of AMR bacteria. Similarly, Mangal *et al*. (2018) [20] documented that the increased use of colistin for treating Gram-negative bacterial (GNB) infections has led to the growing emergence of colistin resistance in numerous locations globally.

Despite extensive research on AMR in clinical settings**,** there is limited information on the prevalence of AMR pathogens in wastewater from hospitals in Enugu, Nigeria, including UNTH and NOHE. Hospital wastewater is a major public health concern because it can act as a reservoir for resistant bacteria, facilitating the broader environmental spread of resistance. This study aims to address this gap by investigating the frequency and mechanisms of colistin resistance in bacteria isolated from the wastewater of UNTH and NOHE. Specifically, by understanding these factors, the study seeks to provide insights into the potential environmental impacts of colistin resistance and guide strategies for managing AMR in hospital settings.

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MATERIALS AND METHODS

Sampling, isolation and identification of bacteria. A total of 20 wastewater samples were collected from four distinct wastewater discharge points within two tertiary hospitals in Enugu metropolis, Nigeria, over a three-month period between January and March 2023. These hospitals were the University of Nigeria Teaching Hospital (UNTH) and the National Orthopaedic Hospital Enugu (NOHE). To ensure a representative sample of the effluent, five independent samples were collected at each discharge point at slightly different time points within a 10-min window to account for potential temporal variation. Each sample was coded numerically (1-5) for identification and stored directly in 250 ml pre-sterilized plastic containers. The four discharge points were designated as follows: NOHE Female Medical Ward (P1), NOHE Amenity Ward (P2), UNTH Post Natal Ward (P3), and UNTH Male Medical Ward (P4).

To maintain sample integrity during transport to the laboratory, the samples were immediately placed in a cooler with ice packs, maintaining a temperature between 2°C and 8°C. Transport time did not exceed two hours. Temperature logs were maintained (every 30 min) for the cooler to monitor conditions throughout transport. Upon arrival at the laboratory, the samples were processed immediately. All laboratory equipment was pre-sterilized, and personnel followed strict aseptic techniques to prevent cross-contamination.

A ten-fold serial dilution of each wastewater sample was carried out using the method described by Hedges *et al*. (2002) [21]. Then, 0.1 mL of each diluted sample (10^{-4} dilution) was dispensed into sterile Petri dishes. Molten, cooled MacConkey agar (HiMedia Laboratories®, India) was added to each plate and gently swirled to ensure even distribution of the sample. The plates were then incubated at 37°C for 24 hours under aerobic conditions in a constant temperature incubator. Following incubation, bacterial colonies were inspected for morphology (shape, size, and color). Isolates were subsequently Gram-stained to categorize them as Gram-positive (purple) or Gram-negative (pink). A series of biochemical tests (Table 1)**,** including Catalase, Oxidase, Nitrate, Hydrogen Sulfide Production, Gas Production, Lactose utilization**,** Glucose Fermentation, Pyocyanin Production, Prodigiosin production, Swarm, and Coagulase tests, were employed to further characterize the bacterial isolates.

Table 1. Biochemical tests used for bacterial identification

Biochemical Test	Purpose	Positive indication	Negative indication	Bacterial species tested
Catalase	To detect enzyme catalase	Gas bubbles from hydrogen peroxide breakdown	No gas bubbles	All isolates
Oxidase	To detect enzyme cytochrome oxidase	Rapid color change to purple or blue	No color change	*Pseudomonas aeruginosa*
Nitrate	To detect nitrate reduction	Gas bubble formation/red color change	No gas bubbles/no color change	All isolates
Hydrogen Sulfide Production	To detect hydrogen sulphide production	Blackening of culture medium	No blackening	*Salmonella* spp., *Proteus* spp.
Gas Production	To detect gas production during fermentation	Bubbles in inverted fermentation tube	No bubbles	All isolates
Lactose Fermentation	To detect lactose fermentation	Color change (yellow to red/pink)	No color change	*Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp.
Glucose Fermentation	To detect glucose fermentation	Color change (yellow to red/pink)	No color change	All isolates
Pyocyanin Production	To detect pyocyanin pigment production	Blue/green coloration of medium	No color change	*Pseudomonas aeruginosa*
Prodigiosin Production	To detect prodigiosin pigment production	Red coloration of medium	No color change	*Serratia marcescens*
Swarm	To detect rapid bacterial movement	Hazy appearance across agar plate	Restricted movement around inoculation site	*Proteus* spp.
Coagulase	To detect coagulase enzyme	Clot formation	No clot formation	*Staphylococcus aureus*

Isolates that fermented lactose and exhibited a greenish metallic sheen on MacConkey agar were presumptively identified as *Escherichia coli*. This presumptive identification was confirmed using a panel of additional biochemical tests as described by Fawole and Oso [22], including:

- Indole Production: Positive indicated by a red ring after adding Kovac's reagent.
- Methyl Red: Positive indicated by a red color after adding methyl red indicator.
- Voges-Proskauer: Positive indicated by a red color after adding VP reagents.
- Citrate Consumption: Positive indicated by a blue color change in the medium.
- Sugar Fermentation: Assessed for various sugars with a color change indicating fermentation.
- Triple Sugar Iron (TSI) Agar: Positive indicated by yellow/red color changes and black precipitate.

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- Motility: Positive indicated by diffuse growth from the stab line.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [23]. Briefly, pure bacterial colonies were suspended in sterile saline to achieve a 0.5 McFarland turbidity standard. A sterile swab was dipped into the suspension and used to evenly inoculate the surface of a Mueller-Hinton agar plate. Antibiotic discs (Oxoid, UK) were then applied to the inoculated agar surface using a sterile forceps. The plates were incubated at 35°C for 16-18 hours under aerobic conditions**.** Following incubation, the diameters of the zones of inhibition (ZOIs) around each disc were measured in millimeters (mm) and interpreted according to the breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [24, 25]. Quality control was performed using *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 to ensure the accuracy of the AST results and the potency of the antibiotic discs**.**

The antibiotics tested were ciprofloxacin (CPX, 10 µg), levofloxacin (LEV, 10 µg), gentamicin (GN, 10 µg), ceftriaxone (CRO, 30 µg), trimethoprim/sulfamethoxazole (SXT, 30 µg), azithromycin (AZ, 30µg), meropenem (MP, 10 ug), ceftazidime (CAZ, 10 µg), cefotaxime (CTX, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg), colistin (10 µg), penicillin (10 µg), and vancomycin (10 µg). The selection of antibiotics for testing was based on several key considerations. First, to identify resistance patterns among diverse pathogens in hospital wastewater, a broad spectrum of antibiotics representing various classes was chosen. This spectrum included agents commonly used for treating both Gram-positive bacteria, such as vancomycin, and Gram-negative bacteria, such as fluoroquinolones like ciprofloxacin and levofloxacin, as well as ceftriaxone, ceftazidime, and cefotaxime. Second, the selection also reflected local antibiotic usage patterns. Antibiotics like ciprofloxacin, levofloxacin, gentamicin, ceftriaxone, trimethoprim/sulfamethoxazole, and azithromycin are frequently employed in the local healthcare setting. Understanding resistance patterns to these commonly used drugs is crucial for optimizing patient treatment strategies. Finally, colistin discs were included to directly assess the prevalence of colistin-resistant bacteria within the hospital wastewater.

To ensure active cultures for antimicrobial susceptibility testing, the isolates were sub-cultured on blood agar and MacConkey agar plates and incubated for 18-24 hours. This step was performed immediately before testing to guarantee the viability and potency of the cultures at the time of testing. The isolates were then prepared for antimicrobial susceptibility testing by streaking them onto Mueller-Hinton agar plates.

Phenotypic detection of colistin resistance. A lawn culture of each test isolate was prepared on a Mueller-Hinton agar plate (HiMedia Laboratories®, India). Colistin minimum inhibitory concentrations (MICs) were determined using the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute [23]**.** Briefly, bacterial suspensions were prepared in Mueller-Hinton broth to achieve a turbidity equivalent to a 0.5 McFarland standard. Two-fold serial dilutions of colistin were prepared in Mueller-Hinton broth in a 96-well microtiter plate, and 100 µL of the bacterial suspension was added to each well. The plates were incubated at 37°C for 18-24 hours. The MIC was recorded as the lowest concentration of colistin that inhibited visible bacterial growth. *Escherichia coli* ATCC 25922 was included in each batch of tests as a quality control strain.

The cultures were incubated overnight at 37°C under aerobic conditions. Isolated colonies were then sub-cultured on blood agar and MacConkey agar and incubated for 18–24 hours at 37°C under aerobic conditions. Colistin MICs were determined using the SensiTest™ Colistin (Liofilchem, Italy), a standardized commercial broth microdilution assay designed for accurate MIC testing of colistin. Colistin susceptibility breakpoints of ≤ 2 mg/L and a resistant breakpoint of > 2 mg/L [24] were established for *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2020). Isolates with a colistin MIC > 2 mg/L were considered colistin resistant.

Detection of mobile colistin resistance genes. Cell lysates were prepared by boiling bacterial suspensions at a concentration of 1.0 McFarland standard for 5 minutes and were used as templates for Polymerase chain reaction (PCR). Positive and negative controls were included in the PCR reactions to ensure the accuracy and reliability of the results. The positive control consisted of purified DNA containing the target gene sequence. This control verified that all PCR components were functioning correctly and that amplification was possible. The negative control lacked the target DNA template and contained all other PCR reagents**.** The negative control monitored for contamination throughout the PCR process. Isolates were screened for the mobile colistin resistance genes *mcr-1* to *mcr-3* via a multiplex PCR protocol to ensure the specificity and efficiency of the process [26]. The annealing temperature was set at 60°C based on previously published protocols. Primer concentrations were optimized according to the manufacturer's recommendations.

Primer Design and Validation. Primer specificity was verified using the Basic Local Alignment Search Tool (BLAST) against the NCBI nucleotide database [26] to ensure accurate targeting of the *mcr* genes and minimize the potential for non-specific amplification. The BLAST analysis confirmed that the selected primers were highly specific for the target *mcr* genes. The annealing temperature was set at 60°C, and the cycle number was fixed at 35 cycles as determined by optimization experiments.

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The primer pairs were synthesized by Inqaba Biotec™, Pretoria, South Africa, as displayed in Table 2.

PCR amplification. The PCR reaction mixture had a final volume of 25 µl, containing 12.5 µl of master mix (New England Biolabs ®), 1 µl of each 10 µM primer, 2 µl of DNA template, and 8.5 µl of nuclease-free water. The PCR protocol included initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 45 seconds. A final elongation step was performed at 72°C for 7 min, and a holding temperature of 10°C was used to complete the amplification process. The PCR reactions were performed using a Bio-Rad C1000 Touch™ Thermal Cycler **(**Bio-Rad Laboratories, Inc., Hercules, CA, USA) with Bio-Rad CFX Manager™ software, ensuring accuracy and reproducibility of the results.

Table 2. Primers used for multiplex PCR detection of *mcr* genes

Gene Target	Primer	Sequence (5' to 3')	Amplicon Size (bp)	Reference
mcr-1	Forward	AGTCCGTTTGTTCTTGTGGC	320	[26]
	Reverse	AGATCCTTGGTCTCGGCTTG		
mcr-2	Forward	CAAGTGTGTTGGTCGCAGTT	500	[26]
	Reverse	TCTAGCCCGACAAGCATACC		
mcr-3	Forward	AAATAAAAATTGTTCCGCTTATG	500	[26]
	Reverse	AATGGAGATCCCCGTTTTT		

Note: All primers were synthesized by Inqaba Biotec™, Pretoria, South Africa

Statistical analysis. Descriptive statistics were used to summarize the data. Continuous variables were presented as means and standard deviations (SD) or medians and interquartile ranges (IQR)**,** as appropriate, while categorical variables were presented as frequencies and percentages. Data were visually presented using tables and bar charts. The chi-square test was used to assess associations between categorical variables. One-way analysis of variance (ANOVA) was used for comparisons of continuous variables among three or more groups. All statistical analyses were performed using SPSS software (version 23.0; IBM Corp., Armonk, NY, USA). A *P*-value of less than 0.05 was considered statistically significant.

Ethical considerations. Ethical approval for this research was obtained from the Health Research Ethics Committees of the National Orthopaedic Hospital Enugu (NOHE) (approval number 6.313/IV/2023/09/033; date of approval: September 28, 2023) and the University of Nigeria Teaching Hospital (UNTH) (approval number NHREC/05/01/2008B-FWA00002458-1RB00002323; date of approval: October 18, 2023).

RESULTS

Isolation and identification of bacteria. A total of 65 bacterial strains were isolated from the 20 wastewater samples. The distribution of bacterial isolates by hospital ward is shown in Table 3. Among the isolates, *Klebsiella* spp. (n = 16, 24.6%) and *E. coli* (n = 6, 9.2%) were the most prevalent Gram-negative bacteria. *S. aureus* (n = 8, 12.3%), coagulase-negative staphylococci (n = 6, 9.2%), and *Enterococcus faecalis* (n = 6, 9.2%) were the most prevalent Gram-positive bacteria. *Proteus mirabilis* was the least prevalent, with only 2 (3.1%) isolates identified from a single ward. Other isolates included *Enterobacter* spp. (n = 6, 9.2%), *P. aeruginosa* (n = 5, 7.7%), *Salmonella* spp. (n = 3, 4.6%), *Serratia marcescens* (n = 3, 4.6%), and *Streptococcus pneumoniae* (n = 4, 6.2%). All isolates underwent standard identification procedures, including following established protocols. The identification results were consistent with the expected biochemical profiles for each bacterial species (Tables 4 and 5)**.**

Antibiotic susceptibility profiles of tested bacteria. The susceptibility profile of the tested bacteria, as presented in Tables 6 and 7, highlights significant differences in resistance patterns among the species tested, with statistical significance determined using ANOVA and chi-square tests ($P < 0.05$). Among the results obtained, *E. coli* strains exhibited resistance to septrin (81%), ceftriaxone (81%), ceftazidime (81%), colistin (75%), meropenem (69%), and cefotaxime (100%). *E. coli's* average resistance across all tested antibiotics was approximately 66.4%. *Pseudomonas aeruginosa* displayed the highest average resistance at 78.2% across all tested antibiotics. *Klebsiella* spp. exhibited an average resistance rate of approximately 68.9%, while *Serratia marcescens* had the lowest average resistance at 48.1%. More than half of the test isolates were resistant to gentamicin, azithromycin, amoxicillin-clavulanic acid, and septrin, whereas more than 60% were sensitive to levofloxacin.

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Table 3. Distribution of bacterial isolates from hospital wastewater samples

Bacterial isolate	Number isolated (%)	Source ward
Klebsiella spp	16 (24.6)	P1, P2, P3 and P4
Escherichia coli	6 (9.2)	P1, P2, P3 and P4
Enterobacter spp.	6 (9.2)	P1, P2, P3
Pseudomonas aeruginosa	5 (7.7)	P2, P3
Salmonella spp.	3 (4.6)	P2, P3
Proteus spp.	2 (3.1)	P1
Serratia marcescens	3 (4.6)	P1 and P4
Staphylococcus aureus	8 (12.3)	P1, P2, P3 and P4
Coagulase-ve staphylococci	6 (9.2)	P1, P2, P3 and P4
Streptococcus pneumoniae	4 (6.2)	P1, P4
Enterococcus faecalis	6 (9.2)	P1, P2, P3 and P4
Total	65	

P1=NOHE Female Medical Ward; P2=NOHE Amenity Ward; P3=UNTH Post Natal Ward; P4=UNTH Male Medical Ward.

Table 4. Biochemical characteristics of isolated Gram-negative bacteria

Isolate	Gram Reaction	Catalase	Oxidase	Nitrate Reduction	Indole Production	Methyl Red	Voges-Proskauer	Citrate Utilization	H_2S Production	Gas Production	Lactose Fermentation	Glucose Fermentation	Pyocyanin Production	Prodigiosin Production	Swarming Motility
Klebsiella spp.	-	+	-	+	-	-	+	+	-	+	+	+	-	-	-
Escherichia coli	-	+	-	+	+	+	-	-	-	+	+	+	-	-	-
Enterobacter spp.	-	+	-	+	-	-	+	+	-	+	+	+	-	-	-
Pseudomonas aeruginosa	-	+	+	+	-	-	-	+	-	-	-	-	+	-	+
Salmonella spp.	-	+	-	+	-	+	-	+	+	-	-	+	-	-	+
Proteus mirabilis	-	+	-	+	-	-	+	+	+	-	-	+	-	-	+
Serratia marcescens	-	+	-	+	-	-	+	+	-	-	-	+	-	+	+

Note: + = positive reaction; - = negative reaction.

Table 5. Biochemical characteristics of isolated Gram-positive bacteria

Isolate	Gram Reaction	Catalase	Coagulase	Oxidase	Nitrate Reduction	Indole Production	Methyl Red	Voges-Proskauer	Citrate Utilization	H_2S Production	Gas Production	Lactose Fermentation	Glucose Fermentation
Staphylococcus aureus	+	+	+	-	+	-	+	+	+	-	-	-	-
Coagulase-negative staphylococci	+	+	-	-	+	-	-	+	-	+	+	+	+
Streptococcus pneumoniae	+	-	-	-	-	-	-	+	-	-	+	+	+
Enterococcus faecalis	+	+	-	-	+	-	-	+	-	-	+	+	+

Note: + = positive reaction; - = negative reaction.

Prevalence of colistin resistance genes in Gram-negative isolates. The occurrence of resistance to colistin and the prevalence of corresponding resistance genes in Gram-negative isolates from hospital wastewater are shown in Fig. 1 and Fig. 2, respectively. The mobile colistin resistance genes investigated were *mcr-1*, *mcr-2*, and *mcr-3*. Among the 6 colistin-resistant *E. coli* isolates tested, 1 (17%) harbored the *mcr-1* gene, while 5 isolates (83%) harbored the *mcr-3* gene. All 12 colistin-resistant *Klebsiella* isolates (100%) harbored the *mcr-1*, *mcr-2*, and *mcr-3* genes. Among the 5 colistin-resistant *Pseudomonas aeruginosa* isolates tested, all 5 (100%) harbored the *mcr-1* gene, while 3 (60%) harbored the *mcr-2* gene and 4 (80%) harbored the *mcr-3* gene.

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Table 6. Antibiotic resistant profiles of Gram-negative bacterial isolates (%)

Antibiotic	*Kl*	*Ec*	*Eb*	*Pa*	*Sal*	*Pm*	*Sm*
Septrin	81	80	83	80	100	100	67
Gentamycin	62	40	33	80	33	0	33
Ciprofloxacin	31	60	33	100	67	50	0
Levofloxacin	26	40	33	40	0	50	33
Azithromycin	44	40	67	80	67	100	67
Meropenem	69	80	83	20	100	50	67
Ceftriaxone	81	80	83	100	100	100	100
Ceftazidime	81	80	83	100	100	100	67
Cefotaxime	100	80	67	100	67	50	100
AMC	74	60	83	60	67	50	0
Colistin	75	83	0	100	0	0	0

Kl = *Klebsiella* spp.; Ec = *E. coli*; Eb = *Enterobacter* spp.; Pa = *Pseudomonas aeruginosa*; Sal = *Salmonella* spp.; Pm = *Proteus mirabilis*; Sm = *Serratia marcescens*; AMC = Amoxicilin-Clavulanic acid

Table 7. Antibiotic resistance profiles of Gram-positive bacterial isolates (%)

Antibiotic	*Sa*	CoNS	*Sp*	*Ef*
Septrin	100	83	75	67
Gentamycin	50	67	75	100
Ciprofloxacin	12	33	25	50
Levofloxacin	0	67	100	33
Azithromycin	67	33	0	0
Meropenem	88	83	100	100
Ceftriaxone	67	50	75	50
Ceftazidime	40	83	100	83
Cefotaxime	88	100	100	67
AMC	67	100	75	100
Colistin	40	83	25	100

Sa = Staphylococcus aureus; CoNS = Coagulase-negative staphylococci*; Sp = Streptococcus pneumoniae; Ef = Enterococcus faecalis*; AMC = Amoxicilin-Clavulanic acid

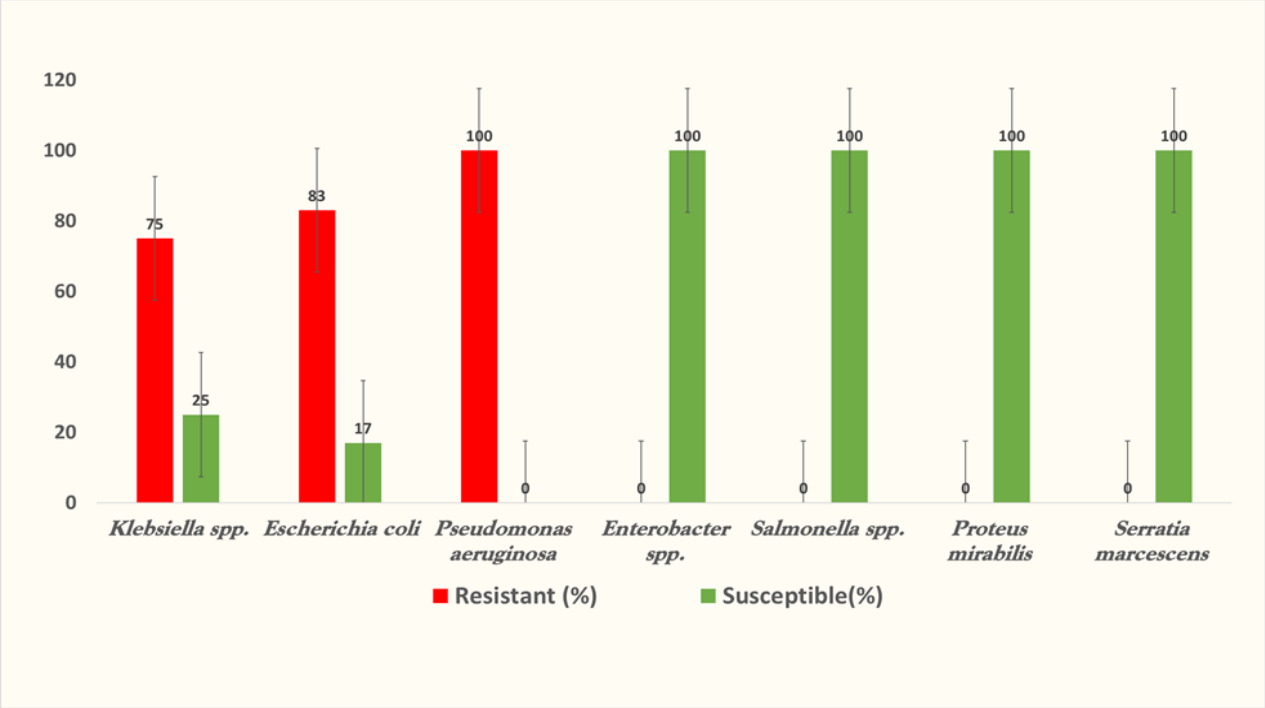

Fig. 1. Prevalence of colistin resistance profile among test isolates

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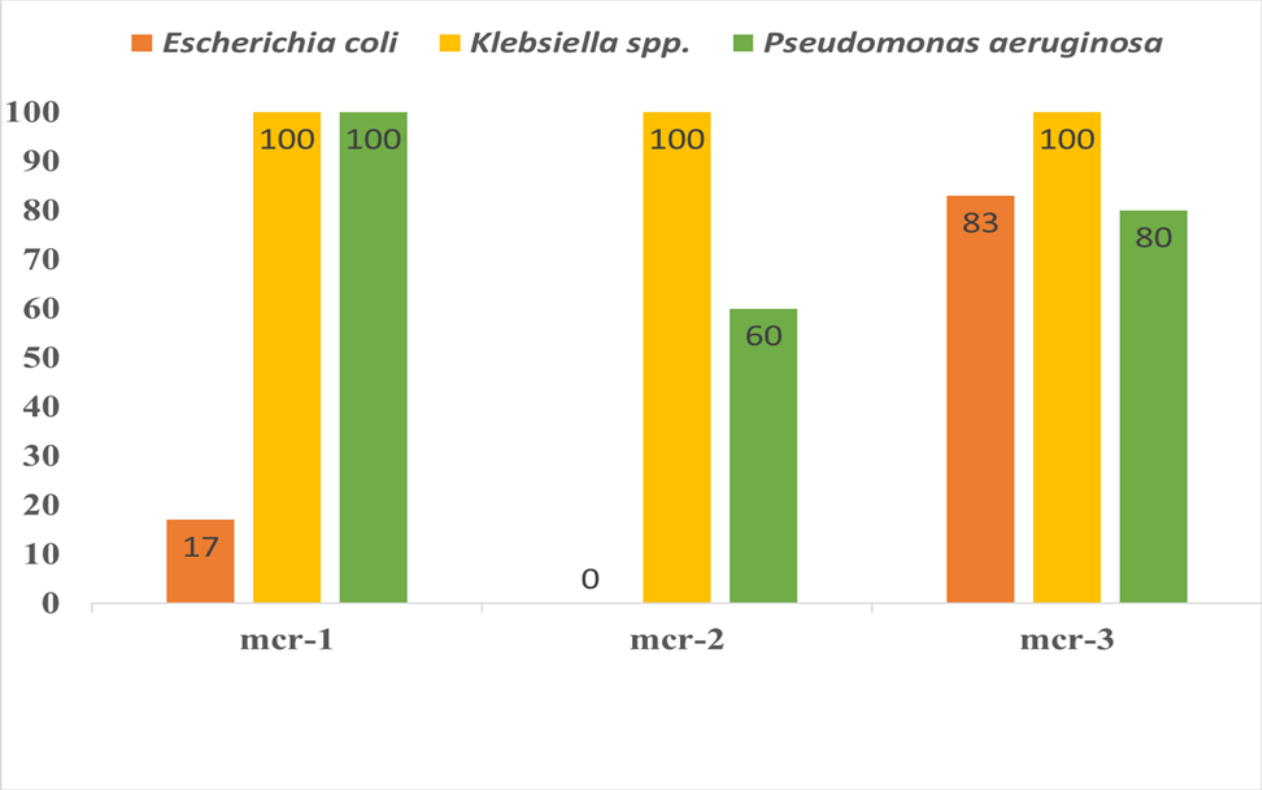

Fig. 2. Prevalence of colistin resistant genes among the test isolates

DISCUSSION

The rise and dissemination of plasmid-borne, mobile colistin resistance genes (*mcr*) poses a significant threat to the effectiveness of colistin, which is considered a last-resort antibiotic for treating severe infections caused by multidrug-resistant and extensively drug-resistant Gram-negative bacteria (GNB) [26, 27].

In our study, 65 bacterial strains were isolated from the 20 samples of hospital effluents collected, with *Klebsiella* spp. identified as the most prevalent bacterial isolate, representing 24.6% of the total isolates. This was followed by *S. aureus* (12.3%) and *E. coli* (9.2). Our findings align with previous research by Mcgann *et al*. (2016) and Villar *et al*. (2018), which also reported *Klebsiella* spp. and *S. aureus* as common contaminants in hospital wastewater [28, 30]. Furthermore, our study provides new insights into the specific prevalence rates within different wards, highlighting the ubiquity of these pathogens in our sampled healthcare settings.

Savin *et al*. (2020) emphasized the concern over the presence of *Klebsiella* spp., *E. coli*, and other pathogenic bacteria in hospital effluents due to their association with nosocomial infections and their potential to acquire resistance to multiple antibiotics, including carbapenems [31]. Our study supports these concerns and further provides detailed prevalence data, revealing a higher incidence of *Klebsiella* spp. compared to the previous study. This difference suggests a potential increase in the prevalence of this pathogen, possibly due to varying infection control practices across different wards.

The high prevalence of these pathogens in various hospital wards, as reported by Savin *et al*. (2020) [31], highlights the risk of transmission and dissemination of antibiotic-resistant strains within the healthcare environment and emphasizes the need for stringent infection control measures, robust wastewater management, and surveillance programs to prevent their dissemination [32, 33].

Our study has some important limitations. First**,** the analysis was based on a relatively small number of samples collected from only two hospitals, which restricts how broadly the findings can be applied to other healthcare facilities. Second, there is a possibility of bias in the results of this study. Specifically, selection bias could have occurred during sample collection, and variations in infection control practices across different hospitals might have influenced the data. Additionally**,** information bias could arise from inconsistent reporting or documentation of infection control measures. To address these issues and improve the generalizability of future research, we recommend conducting multicenter studies with standardized sampling protocols and larger sample sizes. These strategies would help reduce bias and provide more comprehensive and reliable data.

The antibiotic susceptibility test findings of this investigation revealed a concerning prevalence of resistance to commonly prescribed antibiotics across

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various bacterial species. For instance, resistance to septrin was found in 81% of *Klebsiella* spp., 80% of *E. coli*, and 80% of *P. aeruginosa* isolates. Similarly, gentamicin resistance was observed in 62% of *Klebsiella* spp., 40% of *E. coli*, and 80% of *P. aeruginosa* isolates. Resistance to ceftriaxone was notably high, with 81% of *Klebsiella* spp., 80% of *E. coli*, and 100% of *P. aeruginosa* isolates exhibiting resistance. *Klebsiella* spp., *Enterobacter* spp., and *P. aeruginosa* displayed high resistance to a broad range of antibiotics, including third-generation cephalosporins and carbapenems, highlighting their role as significant nosocomial pathogens with multidrug-resistant capabilities.

The emergence of resistance to critical antibiotics, especially last-resort options like colistin and carbapenems, is highly concerning. This study revealed that *P. aeruginosa*, a notorious nosocomial pathogen that was listed as a Priority 1 pathogen by the WHO in 2017, displayed 100% resistance to colistin and third-generation cephalosporins, which, according to Sib *et al*. (2020) [10], indicates limited treatment options for infections caused by these organisms. Similarly, *Salmonella* spp., *S. pneumoniae*, and *E. faecalis* exhibited complete resistance to meropenem, underscoring the challenge of combating multidrug-resistant pathogens in clinical settings [12]. The high prevalence of antibiotic-resistant bacteria, especially those resistant to last-resort antibiotics like colistin, poses significant challenges for patient management and infection control. This underscores the urgent need for alternative treatment strategies and the development of new antibiotics to combat resistant strains. Effective infection control measures, including strict hygiene practices, surveillance programs, and antibiotic stewardship, are crucial to curb the spread of these resistant pathogens within healthcare settings.

Colistins are cationic antimicrobial agents that target the phosphate component of bacterial lipopolysaccharide (LPS), causing cell death by disrupting the negative charge of the outer membrane [15]. Colistin resistance is primarily caused by LPS covalent modification, which results in reduced affinity between LPS and colistin [14]. Phenotypically, varying levels of colistin resistance were observed among different bacterial isolates [33]. *E. coli* and *Klebsiella* spp. demonstrated a high prevalence of colistin resistance, with 83% and 75% of isolates, respectively, being resistant. *P. aeruginosa* exhibited 100% resistance to colistin. Studies by Berendonk *et al*. (2015) [34] and Ma *et al*. (2019) [27] have also highlighted a rise in colistin resistance, particularly among Gram-negative pathogens associated with healthcare-acquired infections. Our results further support this trend, suggesting a potential limitation in colistin's effectiveness in treating these infections in our healthcare setting. According to Olaitan *et al*. (2014) [35], these isolated bacteria have been commonly associated with healthcare-associated infections such as pneumonia, urinary tract infections, and bloodstream infections, which makes the high prevalence of colistin resistance observed in them a serious concern, as it underscores the limited treatment options available [36]. In contrast, the analysis of the samples revealed that *Salmonella* spp., *P. mirabilis*, and *S. marcescens* did not exhibit colistin resistance.

The presence of mobile colistin resistance genes (*mcr-1*, *mcr-2*, and *mcr-3*) in isolates from this study highlights a particularly alarming aspect of antibiotic resistance: the potential for these genes to be readily transferred between bacterial species. Unlike changes within the bacteria's own DNA, *mcr* genes reside on plasmids, tiny independent rings of genetic material. These plasmids act like portable toolboxes, which are readily shared between bacteria through a process called horizontal gene transfer (HGT). HGT essentially functions as a shortcut, allowing bacteria to acquire new abilities, including antibiotic resistance, from their neighbors [37].

All tested isolates of *Klebsiella* spp. and *P. aeruginosa* harbored *mcr* genes, with *Klebsiella* spp. isolates harboring all three *mcr* genes (*mcr-1*, *mcr-2*, and *mcr-3*). This finding, according to Yang *et al*. (2020) [38], suggests a remarkable dissemination of colistin resistance mechanisms within these species and the potential for cumulative effects that confer high-level resistance to colistin. Furthermore, Du *et al*. (2016) highlighted that the high prevalence of *mcr* genes among *Klebsiella* spp. isolates, in particular, signifies the role of this pathogen as a reservoir for antimicrobial resistance genes, which can be transmitted horizontally to other bacteria [39]. Implementing stricter antibiotic stewardship programs and developing novel strategies to disrupt HGT mechanisms are essential steps to curb the emergence and spread of MDR bacteria [40].

This study highlights the rising prevalence of colistin resistance among bacteria in hospital wastewater, particularly in *Klebsiella* spp., *E. coli*, and *P. aeruginosa*. Specifically, colistin resistance was observed in 75% of *Klebsiella* spp., 83% of *E. coli*, and 100% of *P. aeruginosa* isolates. The presence of *mcr* genes (*mcr-1*, *mcr-2*, *mcr-3*) in these isolates emphasizes the potential for horizontal gene transfer, posing a significant challenge for infection control within healthcare settings in Enugu Metropolis.

The detection of colistin-resistant bacteria in hospital wastewater effluents stresses the critical role of wastewater as a reservoir for resistant bacteria and their genes. Effective wastewater management is therefore essential to prevent the dissemination of these resistance genes into the broader environment. To mitigate this risk, it is crucial to implement stringent wastewater treatment protocols, regularly monitor wastewater for antibiotic-resistant bacteria, and develop policies aimed at controlling the spread of resistance genes from hospital effluents to the community. These measures will help reduce the environmental contamination and transmission of resistance genes, which is essential for supporting the fight against antibiotic resistance in both healthcare and community settings.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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