International Journal of Pharmacognosy and Life Science

**E-ISSN:** 2707-2835
**P-ISSN:** 2707-2827
www.pharmacognosyjournal.com
IJPLS 2023; 4(2): 120-127
Received: 04-09-2023
Accepted: 03-10-2023

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# Antibiotic resistance profile of clinical importance biofilm forming extended spectrum beta-lactamase and carbapemase phenotype in gram-negative bacteria isolates

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**DOI:** https://doi.org/10.33545/27072827.2023.v4.i2b.97

**Abstract**
The occurrence of extended Spectrum β-lactamases (ESBL) and carbapenemase enzymes associated with clinically relevant biofilm forming Gram-negative bacteria (GNB) pathogens is increasing over time, posing major health risks. This study was conducted with the aim to report an in-depth of the emergence of biofilm forming GNB harboring ESBL and carbapenemase phenotype circulating in patients in Tertiary hospital in South-eastern Nigeria. A total of three hundred (300) urine samples were subjected to routine microbiological analysis. The Double Disk Synergy Test (DDST) and the Modified Hodge Test (MHT) were used to detect ESBL production and carbapenem resistance, respectively. Detection of biofilm production was performed using microtitre plate-based assay. The Kirby-Bauer disk diffusion method was used to determine antibiogram investigations of biofilm producing ESBL and carbapenem resistant GNB. Result of isolation and characterization revealed overall frequency of 217(72.3%) GNB comprising of highly prevalence *Pseudomonas aeruginosa* 109(36.3%) followed by *Klebsiella pneumoniae* 67(22.3%) and *Escherichia coli* 41(13.7%). Phenotypic ESBL and carbapenem resistant producing GNB accounted for 55(25.3%) and 15(6.9%) respectively. Overall detection rate of biofilm forming ESBL and carbapenem-resistant producing GNB was 12 (21.8%) and 7 (58.3%) respectively. Resistance was found in a high percentage of the isolates to Amoxycillin-Clavulanic acid 100%, Ceftriaxone 100%, Cefotaxime 75.5%, amikacin 50.0%. Our findings shows that the pattern of resistance phenotype between this GNB may cause the expansion of MDR to XDR strains that makes selecting an appropriate treatment challenging for any GNB disease condition. This study recommends that intensified global effort are needed to prevent the dissemination of biofilm producing GNB harboring β-lactamase resistant determinant and eradicate the hospital-borne bacteria that are significantly causing a dramatic increase in mortality. Further genomic study, as well as routine monitoring of biofilm formation capacity and antimicrobial resistance profiles of GNB isolates, are required to determine the precise relationship between these two parameters.

**Keywords:** Gram-negative**,** biofilm forming, Extended spectrum beta-lactamase, carbapenemase

## Introduction
Gram-negative bacteria (GNB) are ubiquitous, and commonly prevalent in nature, causing potentially fatal illnesses in humans (Owusu *et al.*, 2023) [1]. This class includes *Klebsiella* species, *Escherichia coli*, *Pseudomonas* species, *Proteus* species, *Acinetobacter baumannii*, *Yersinia pestis*, and *Chlamydia trachomatis* etc., are connected with health care, and are important cause of infection with a high antibiotic resistance burden (Yusof *et al.*, 2022; Ogba *et al.*, 2022; Nomeh *et al.*, 2023; Mustafai *et al.*, 2023; Egwu *et al.*, 2023) [2-6].
Under severe conditions, GNB form micro-colonies to aid their mutual survival in the same niche (Peter *et al.*, 2022) [7] as biofilm producers.
Biofilm produced by GNB serve as an organized colonies of micro-organism embedded in a self-produced matrix of extracellular polymeric substances that is attached or connected to a surface. (Achek *et al.*, 2020) [8]. The biofilm formation is classified into three stages: Initial microbial cell attachment, biofilm maturation and microbial cells dispersion (Achek *et al.*, 2020; França *et al.*, 2016) [8, 9].

Earlier published article revealed that a prevalence rate of 60-100% in chronic laceration or wounds and other infections, biofilm is one of the most intricate components associated in human infection (Peter *et al.*, 2022; Puca *et al.*, 2021) [7, 10].

The persistence nature of biofilm infections are debilitating to patients leading to prolong hospitalization and hope recuperation fading (Peter *et al.*, 2022; Puca *et al.*, 2021) [7, 10].

The protective layer of biofilm protects GNB against the action of antibiotics and the human immune system. The clinical use of antibiotics as empirical therapy to eliminate biofilm-forming GNB is regarded as both the source of developing drug resistance and the remedy to infections.

β-Lactam antibiotics are the most often used drugs for treating bacterial infections. β-lactam antibiotics abuse and improper use are the leading causes of GNB resistance globally (Gebremedhin *et al.*, 2023; El-Masry *et al.*, 2023) [11, 12], both in the community and in clinical settings, and are linked with significant mortality and morbidity (Ogba *et al.*, 2022; Nomeh *et al.*, 2023; Gebremedhin *et al.*, 2023; Joseph *et al.*, 2023) [3, 4, 11, 13].

The development of β-lactamases that enzymatically hydrolyze β-lactam core of the antibiotics is the most common mechanism by which GNB acquire resistance to these antibiotics (Joseph *et al.*, 2023) [13].

The hydrolytic capability of the enzyme target penicillins, aztreonam, cephalosporins, but not carbapenems or cephamycins and are inhibited by β-lactamase inhibitors such as sulbactam, clavulanic acid, tazobactam, (Joseph *et al.*, 2023) [13].

Currently, the most prevalent β-lactamases discovered in GNB are extended spectrum β-lactamases (ESBLs) and carbapenemases, mainly in *Escherichia coli*, *Pseudomonas* species, and *Klebsiella pneumoniae* (Ogba *et al.*, 2022; Nomeh *et al.*, 2023) [3, 4].

GNB-producing ESBLs are among the World Health Organization's enlisted group of critical priority pathogens for novel antibiotic research development (Joseph *et al.*, 2023) [13].

The most frequent ESBL genotypes are $bla_{TEM}$, $bla_{SHV}$, and $bla_{CTX\text{-}M}$ (Owusu *et al.*, 2023) [1], which are mostly carried on plasmids and frequently carry genes that mediate resistance to other classes of antibiotics such as trimethoprim-sulfamethoxazole, tetracycline fluoroquinolones, aminoglycosides, and, further limiting treatment options for ESBL-associated infections (Owusu *et al.*, 2023) [1].

However, Carbapenems, on the other hand, are one of the few last-line antibiotics for biofilm-forming bacteria that produce ESBL, particularly for the treatment of critically ill individuals or those with a Gram-negative infection that is resistant to the majority of antibiotics (Mustafai *et al.*, 2023) [5]. However, Gram negative bacterial infections that produce carbapenemase (CP) and are carbapenem-resistant chosen due to the overuse of carbapenems to treat biofilm and ESBL-positive pathogens (Mustafai *et al.*, 2023; Idrees, *et al.*, 2022) [5, 14]. In hospitals throughout South and Southeast Asia and South eastern Nigeria, carbapenem resistance is common in Gram-negative bacteria (Ogba *et al.*, 2022; Nomeh *et al.*, 2023; Mustafai *et al.*, 2023; Idrees and Saeed, 2021) [3, 4, 5, 15] while hospital-based research in Sub-Saharan African countries found a range of 9% to 60% ofcarbapenemase-producing bacterial isolates (Gebremedhin *et al.*, 2023; Wangai *et al.*, 2019) [11, 16]. ESBL and CPE have been progressively reported in clinical sources around the world during the last decade (Ogba *et al.*, 2022; Nomeh *et al.*, 2023; Mustafai *et al.*, 2023; Egwu *et al.*, 2023; Egwu *et al.*, 2021) [3-6, 17]. On top of this, such resistance genotype can disseminate by vertical transfer or by horizontal circulation of mobile genetic elements within the biofilm communities and between humans. However, data collection and surveillance of biofilm forming GNB harboring ESBL and carbapenemase genes are frequently scarce, especially in low- and middle-income nations including several African countries. This study was conducted to give an in-depth account of the emergence of biofilm forming GNB harboring ESBL and carbapenemase genes circulating in patients in tertiary hospital in South eastern, Nigeria.

## Materials and Methods

### Study Area

After ethical clearance approval and patients consent sorted. The research was carried out at Alex Ekwueme Federal Teaching Hospital Abakaliki, Ebonyi State Abakaliki, located at latitude 6.3231ºN and longitude 8.1121ºE. Ebonyi State, located 64 kilometers southeast of Enugu in southeastern Nigeria. The majority of the residents are Igbo rice and salt producers. The metropolis of Abakaliki is located in the eastern section of the country at latitude 6.3°E and longitude 8.1°N (Adibe-Nwafor *et al.*, 2023) [18].

### Sample processing

Aseptically, a total of three hundred urine samples were collected from hospitalized patients at AE-FUTHA. A loopful of each colony was streaked aseptically on solidified MacConkey agar and Cetrimide agar and CLED agar plate (Hi-Media, India). By plating onto nutrient agar (Hi-Media, India), all distinct colonies were purified. Isolates were classified according to on colonial morphology (consistency, color, and texture), microscopic techniques (Gram staining, and motility test), and biochemical properties such as oxidase test, citrate test, indole test, triple sugar iron test, Voges-Proskaeur test, methyl red test, and carbohydrate fermentation tests as described by Cheesbroough (2006) [19].

### Biofilm formation assay

All isolates' bacterial adhesion was evaluated using a microtitre plate-based assay, which was adapted from a previously published method (O'Toole, 2011) [20]. To put it briefly, a few colonies of each isolate were taken from fresh cultures and placed in tubes containing 3 mL of Tryptic Soy Broth (TSB), which were then incubated for 24 hours at 37 °C. After the incubation period, 100 µL of each bacterial suspension was transferred to a 96-well microtiter plate, and the number of cells in each culture was counted and corrected to 0.5 McFarland ($1.5 \times 10^8$ CFU/mL). A well-known biofilm-forming strain, *Pseudomonas aeruginosa* ATCC® 27853, was included as a positive control in the biofilm assay. Incorporating sterile TSB served as a negative control. At 37°C, the microplates were incubated for 24 hours. Following incubation, the microplates were turned over to extract the bacterial cells in suspension, and they underwent two rounds of distilled water washing. This step greatly reduces background staining by assisting in the removal of stray cells and medium components that might be stained in the subsequent stage. After that, the plates

were left to dry for fifteen minutes at room temperature. The biofilm was then fixed by adding 125 µL of methanol (Scharlau, Barcelona, Spain) to each well and incubating for 15 minutes. Following the removal of methanol and a 10 to 15 minutes room temperature drying period, 125 µL of 1% (v/v) Crystal Violet (CV) (Liofilchem, Roseto degli Abruzzi, Italy) was applied to each well. Following incubation, the microplates were rinsed three to four times with the CV solution removed. Following incubation, the microplates were cleaned three or four times using distilled water, and the CV solution was removed. The plates were then firmly dried on a stack of paper towels to eliminate any remaining cells and stains and was left to dry overnight. A microplate reader was used to measure the optical density at 630 nm (OD630 nm) in order to determine the biofilm biomass. The results indicated the presence of weak, moderate, and vigorous producers (Stepanovic *et al.*, 2007) [21]. 125 µL of 30% (v/v) acetic acid was added every 10-15 minutes.

**Modified Hodge testing**

Based on CLSI breakpoints (CSLI, 2019) [22], bacterial isolates were resistant to imipenem (IPM 10 µg), doripenem (10 µg), meropenem (MEM 10 µg), and ertapenem were subjected for confirmation for carbapenemase production. The Modified Hodge Test (MHT) was used to confirm carbapenemase production in Gram negative bacteria. Mueller-Hinton agar with 0.5 MacFarland standardized suspension of over-night sub-cultured E. coli strain ATCC 25922 was streaked into a Mueller-Hinton agar containing ATCC BAA-1706 as a negative control and ATCC BAA-1705 as a positive control and allowed for confluent growth. A 10 µg ertapenem disk was placed in the center, and each test isolate was streaked from the disk to the edge of the plate. A positive Modified Hodge Test (MHT) was indicated by the presence of a distorted or clover leaf-shaped inhibition zone of the *K. pneumoniae* ATCC BAA-1705 growing along the test organism growth streak within the disk diffusion zone, as recommended by the Clinical and Laboratory Standards Institute methods (CLSI, 2019) [22].

**Screening of extended spectrum beta-lactamase production**

Using the double disk synergy test (DDST), ESBL production was validated phenotypically in only GNB isolates that showed reduced resistance to 2nd and 3rd generation cephalosporins (such as cefotaxime, ceftazidime, and ceftriaxone) (Joseph *et al.*, 2023) [13]. Standardized inoculums of the test isolate adjusted to 0.5 McFarland turbidity standards were aseptically swabbed on the MH agar plates, and an amoxicillin clavulanic acid disc (20/10 µg) was placed in the center of the plate, while cefotaxime (30 µg) and ceftazidime (30 µg) discs were placed 15 mm apart. The plates were incubated at 37ºC for 18-24 hours, and ESBL formation was determined phenotypically by the enlargement of the zone of inhibition of either cephalosporin in the presence of amoxicillin-clavulanic acid compared to its absence, resulting in a dumbbell shape (Joseph *et al.*, 2023) [13].

**Antimicrobial Sensitivity Testing**

Antibiotic susceptibility testing was carried out using the Kirby Bauer disk diffusion method on sterilized Mueller-Hinton agar in compliance with Clinical and Laboratory Standards Institute (CLSI, 2019) [22]. The test isolate's bacteria suspension was produced using 0.5 McFarland standards and seeded on solidified Mueller-Hinton agar. For 5 minutes, the plates were allowed to pre-diffuse. Thereafter, the following antibiotic: Aztreonam (30 µg), Amikacin (10 µg), Amoxycillin-Clavulanic acid (30 µg), Amoxicillin (30 µg), Cefotaxime (30 µg), Cefuroxime (30 µg), Cefoxitin (30 µg), Ceftriaxone (30) µg, Ceftazidime (30 µg), Colistin Sulphate (10 µg), Ciprofloxacin (10 µg), Nalidixic acid (5 µg), Imipenem (30 µg), Ofloxacin (30 µg), Piperacillin (30 µg), Tetracycline (30 µg),trimethoprim Sulphamethoxazole (125/23.75 µg) was impregnated on the inoculated Mueller-Hinton (MH) agar plates and incubated at 37°C for 24 hours. The widths of zones of inhibition were measured after an overnight incubation, and the results were interpreted using Clinical and Laboratory Standards Institute standards (CLSI, 2019; Oke *et al.*, 2020; Uzoije *et al.*, 2021) [22-24].

**Result**

The frequency of isolation of Gram-negative bacteria was 217(72.3%) comprising of highly prevalence *Pseudomonas aeruginosa* 109(36.3%) followed by *Klebsiella pneumoniae* 67(22.3%) while *Escherichia coli* 41(13.7%) had the least occurrence rate as shown in Table 1.

**Table 1:** Frequency of isolation of gram-negative bacteria from urine samples of patients

| Bacteria isolated from urine samples | Occurrence rate (%) |
|---|---|
| *Escherichia coli* | 41(13.7) |
| *Klebsiella* pneumoniae | 67(22.3) |
| *Pseudomonas aeruginosa* | 109(36.3) |
| Total | 217(72.3) |

Extended Spectrum β-lactamases producing Gram-negative bacteria accounted for 55(25.3%) comprising of 27(65.8%), 18(26.8%) and 10(9.2%) from *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively while Carbapenemase β-lactamases producing Gram-negative bacteria accounted for overall detection rate of 15 (6.9%) consisting of *Escherichia coli* 5(12.1%), *Klebsiella pneumoniae* 4(5.9%) and *Pseudomonas aeruginosa* 6(5.5%) as presented in Table 2.

**Table 2:** Frequency of detection of Extended Spectrum β-lactamases (ESBL) and Carbapenemase producing Gram negative bacteria from urine samples of patients

| Bacteria isolated | Extended Spectrum β-lactamases | | Carbapenemase β-lactamases | |
|---|---|---|---|---|
| | Positive (%) | Negative (%) | Positive (%) | Negative (%) |
| *Escherichia coli* | 27(65.8) | 14(51.8) | 5(12.1) | 36(87.8) |
| *Klebsiella pneumoniae* | 18(26.8) | 49(73.1) | 4(5.9) | 63(94.0) |
| *P. aeruginosa* | 10(9.2) | 99(90.8) | 6(5.5) | 103(94.4) |
| Total | 55(25.3) | 162(74.6) | 15(6.9) | 202(93.0) |

In Table 3, overall detection rate of Biofilm forming potential of ESBL producing Gram negative bacteria was 12 (21.8%). *Escherichia coli* comprising of high proportion 6 (66.6%) followed by *Pseudomonas aeruginosa* 3 (30.0%) and *Klebsiella pneumoniae* 3 (16.6%). While non-biofilm formers accounted for 43 (78.1%)

**Table 3:** Biofilm forming potential of ESBL producing Gram negative bacteria isolated from urine samples of patients

| Bacteria isolated | Biofilm former (%) | Non-biofilm formers (%) |
|---|---|---|
| *Escherichia coli* | 6 (66.6) | 21 (77.7) |
| *Klebsiella pneumoniae* | 3 (16.6) | 15 (83.3) |
| *Pseudomonas aeruginosa* | 3 (30.0) | 7 (70.0) |
| Total | 12 (21.8) | 43 (78.1) |

Total number of bacteria = 55

As shown in Table 4 below, the proportion of Biofilm forming Carbapenemase producing Gram negative bacteria isolate accounted for 7 (58.3%) while the frequency of BFCPR-*Klebsiella pneumoniae* accounted for 3 (75.0%), BFCPR-*Escherichia coli* 3 (60%) and BFCPR-*Pseudomonas aeruginosa* 1 (16.6%).

**Table 4:** Biofilm forming Carbapenemase producing gram negative bacteria isolated from urine samples of patients

| Bacteria isolated | Biofilm former (%) | Non-biofilm formers (%) |
|---|---|---|
| *Escherichia coli* | 3 (60) | 2 (40) |
| *Klebsiella pneumoniae* | 3 (75) | 1 (25) |
| *Pseudomonas aeruginosa* | 1 (16.6) | 5 (83.3) |
| Total | 7 (58.3) | 8 (53.3) |

Total number of bacteria = 15

All biofilm forming Gram-negative bacteria were 100% resistant to Trimethoprim-Sulfamethoxazole, Azeteonam, Tetracycline, piperacillin, Amoxycillin-Clavulanic acid, Amoxicillin, Cefuroxime, Cefoxitin. Biofilm-forming isolate were susceptible to amikacin, ceftriaxone and ofloxacin within the range of 11.1%-50.0% in Table 5.

**Table 5:** Antibiotic susceptibility pattern of biofilm forming bacteria

| Antibiotic (µg) | *E coli* (N=9) | | *K. Pneumoniae* (N=6) | | *P Aeruginosa* (N=4) | |
|---|---|---|---|---|---|---|
| | R (%) | S (%) | R (%) | S (%) | R (%) | S (%) |
| Amikacin (10) | 6(66.7) | 3(33.3) | 3(50) | 3(50) | 3(75.0) | 1(25.0) |
| Amoxycillin-Clavulanic acid (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Amoxicillin (10) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Cefotaxime (30) | 5(55.6) | 4(44.4) | 6(100) | 0(0.0) | 3(75.0) | 1(25.0) |
| Cefuroxime (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Cefoxitin (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Ceftriaxone (10) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 2(50.0) | 2(50.0) |
| Ceftazidime (30) | 7(77.7) | 2(22.2) | 5(83.3) | 1(16.7) | 4(100) | 0(0.0) |
| Colistin Sulphate (10) | 8(88.9) | 1(11.1) | 3(50) | 3(50) | 4(100) | 0(0.0) |
| Ciprofloxacin (10) | 8(88.9) | 1(11.1) | 6(100) | 0(0.0) | 3(75.0) | 1(25.0) |
| Nalidixic acid (5) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Imipenem (30) | 7(77.7) | 2(22.2) | 3(50) | 3(50) | 4(100) | 0(0.0) |
| Ofloxacin (10) | 5(55.6) | 4(44.4) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Piperacillin (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Tetracycline (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Trimethoprim-Sulfamethoxazole (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |
| Aztreonam (30) | 9(100) | 0(0.0) | 6(100) | 0(0.0) | 4(100) | 0(0.0) |

Key: R-Resistance, S-Susceptible, %-Percentage

## Discussion

There was high rate of isolation of Gram-negative bacteria (GNB) 217(72.3%) in urine samples collected**.**

It is also probable that inadequate or poor infection management, such as abscesses, bloodstream infections, bacteriuria, and so on, as well as patient's hygiene, may have led to a significant increase in the proliferation and incidence of Gram-negative bacteria in urine samples over time.

Disaggregation of the GNB, elucidate higher occurrence rate of *Pseudomonas aeruginosa* 109(36.3%) and strongly substantiate with Rajat *et al.* (2012) [25] and Ogba *et al.* (2023) [3] published higher prevalence rates of 32.1% and 23.8% in Ahmadabad, India and, Abakaliki, Nigeria, respectively, and additional studies detected their presence in clinical samples in Germany (Schäfer *et al.*, 2019) [26].

It should be pointed out that *P. aeruginosa* is a frequently encountered cause of community-acquired infections among individuals with chronic underlying conditions, as well as hospital-acquired infections such as pneumonia, urinary tract infections, and bloodstream infections (BSIs) (Yoon *et al.*, 2021) [27]. In 2020, it was reported as an infectious agent that co-infects people with COVID-19. (Ogba *et al.*, 2023; Yoon *et al.*, 2021) [3, 27].

It should be noted that the proportion of *P. aeruginosa* isolates varies with specimens and clinical conditions, whereas juxtaposing epidemiological data of enterobacteria as in this study may be challenging due to multiple factors that influences the outcome of results such as duration of clinical specimens transported for examination, geo-graphical locations, studied group and type of hospitals.

*K. pnuemoniae* was the second most common GNB which accounted for 67(22.3%) from the studied samples which is a very low percentage occurrence when compared with two previous studies from Eastern Cape Province (ECP) and Pretoria in South Africa that reported 83% and 86% prevalence of *K. pneumoniae* (Mbelle *et al.*, 2020; Ebomah and Okoh, 2020) [28, 29] but also substantiate with low prevalence rate reported in Lebanon 8.3%, Malaysia 14.0%, Nigeria 16.3%, Brazil 25.2% and Ethopia 30.3% (Beyrouthy *et al.*, 2014; Rahman *et al.*, 2018; Nomeh *et al.*, 2022; Ribeiro *et al.*, 2016; Beyene *et al.*, 2019) [30-34].

The heterogeneity in the epidemiology of *K. pneumoniae* from the earlier studies may be attributed to differences in

the size of the sample, population being studied, and methodological variations.

In recent years, *K. pneumoniae* is one of the leading causes of community and hospital-acquired infections, manifesting as urinary tract infections, pneumonia, abscesses, septicaemia, bloodstream infections, particularly in neonates, elderly and immunocompromised individuals (Nomeh *et al.*, 2022; Wang *et al.*, 2020; Sakkas *et al.*, 2019) [32, 35, 36].

From the result section, the least GNB was *E. coli* which accounted for 13.7%. Despite the fact that there appears to be geographical variances in the proportions of the species previously identified in prior studies, our observation is unparalleled with reports in Abakaliki 54.3%, Port Harcourt 49.3%, Yemen 36.6%, (Nomeh *et al.*, 2022; Kpalap *et al.*, 2019; Alsharapy *et al.*, 2018) [32, 37, 38] and few studies conducted have also documented a high predominant of *Escherichia coli* in other human sample (Ugbo *et al.*, 2020; Ugwu *et al.*, 2020) [39, 40]. The overabundance of *E. coli* in urine sample would have been anticipate in the study and these bacteria are generally recognized as the intestinal microbiota of animals, and humans, with virulence strains capable of causing UTIs and other extraintestinal infections (Joseph *et al.*, 2023) [13].

These UPEC strains exhibit virulence characteristics that allow them to colonize human hosts successfully. Furthermore, the low proportion of *E. coli* may imply proper conformance to hygiene practice, which are effective strategies to limit fecal bacteria transfer.

The isolate resistance rate of 33.3-100% was demonstrated against 2nd and 3rd generation cephalosporins.

This outcome is consistent with earlier study in Abakaliki that revealed *E. coli* resistance to ceftazidime 79.5% and ceftriaxone 57.5% (Ugbo *et al.*, 2020) [39]. Meanwhile, in comparable research in Sri Lanka, Fernando *et al.* (2017) [41] found high resistance to ceftazidime 100%, and ceftriaxone 100%, while in Upper Egypt, Hassuna *et al.* (2020) [42] recorded 100% resistance to ceftazidime. Dehbashi *et al.* (2020) [43] also illustrates *P. Aeruginosa* increased resistance level to ceftazidime (44.31%) and cefepime (30.68%). In Sudan, *P. aeruginosa* isolates had the highest number of resistant to 2nd generation cephalosporins 100%, 3rd generation cephalosporins 70.6%, 1st generation cephalosporins 58.9% (Azab *et al.*, 2021) [44] whereas in more than 20% of articles, the rate of 4th generation cephalosporins (cefepime) resistance is considered more than 90% (Amini *et al.*, 2018; Salehi and Amini, 2017) [45, 46]. In yet another study, K. pneumoniae isolates were 100% resistant to ceftriaxone, 95.8% resistant to ceftazidime, and 83.3% resistant to cefepime. (Sakkas *et al.*, 2019) [36]. Ceftazidime 66.7% and Ceftriaxone 92.3% resistance in MDR *K. pneumoniae* has been observed in Ibadan, Nigeria. (Makanjuola *et al.*, 2018) [47]. An earlier study conducted in Saudi Arabia found that 4 ESBL *K. pneumoniae* isolates were completely resistant to ceftazidime, cefepime and ceftriaxone (Azim *et al.*, 2020) [48]. The study conducted by Yasir *et al.* (2018) [49] also revealed that the majority of ESBL isolates are multidrug-resistant (MDR), particularly to first, third, and fourth-generation cephalosporins. Individuals' indiscriminate use and abuse of beta-lactam antibiotics have generated problems in treating microbial infections and disorders caused by these antibiotic-resistant organisms due to ESBL production, according to this study's high incidence of resistance.

Our investigation found GNB bacteria that were 50.0% and 100% resistant to colistin, which could represent the continuing existence of colistin resistance in Abakaliki. (Ogba *et al.*, 2022; Nomeh *et al.*, 2022) [3, 32].

Colistin is a polymyxin that was widely used against Gram-negative bacteria in the past (1940s-1970s) but was discontinued due to nephrotoxic and neurotoxic adverse effects. However, due to the emergence of β-lactamase resistant Gram-negative bacteria that were discovered to be vulnerable or susceptible to polymyxins, this forgotten medication was reintroduced into use in the early 2000s. (Sakkas *et al.*, 2019) [36]. Regretfully, colistin resistance among GNRs that are resistant to carbapenem rose in tandem with the utilization of colistin (Satlin, 2019) [50]. There have been reports from two US. A multicenter clinical laboratory research on carbapenem-resistant *K. pneumoniae* isolates demonstrating 13% and 16% colistin-resistance (Satlin *et al.*, 2017; Rojas *et al.*, 2017) [50, 51]. Furthermore, 2 (50. 0%) and 3 (75.0%) colistin-resistant *E. coli* strains carried chromosomal *mcr*-1 and plasmid-mediated genes respectively (Karki *et al.*, 2021) [52]. According to Qadi *et al.* (2021) [53], *E. coli* and *Klebsiella* resistance to colistin was 33.3% and 31.6% respectively while Alfoiuzan *et al.* (2018) [54] discovered 4.3% and 7.7% resistant by *E. coli* and *Klebsiella* resistance respectively (Alfoiuzan *et al.*, 2018) [54]. In a study of colistin resistance in *E. coli* and *Klebsiella pneumoniae* bacteria obtained from cancer patients, 45.0% of colistin-resistant isolates were also meropenem-resistant. (Zafer *et al.*, 2019) [55]. Prim *et al.* (2017) [56] reported that colistin resistance was present in 0.67% of cases. *Enterobacter cloacae* 4.2% had a greater rate than *Escherichia coli* 0.5% and *Klebsiella pneumoniae* 0.4%. In Abakaliki and Thailand, 100% colistin-resistant *P. aeruginosa* has been reported (Ogba *et al.*, 2022; Pungcharoenkijkul *et al.*, 2020) [57, 58]. Based on previous research identifying this gene as a significant target for the development of colistin resistance in enterobacteria, the high incidence of colistin resistance in this environment or examined area confirms the role of probable alterations in the mgrB gene (Bonura *et al.*, 2015; Cannatelli *et al.*, 2014) [59, 60]. Notably, ST392 KPC-2-producing *K. pneumoniae* encoding the mcr-1 gene, which confers colistin resistance, has been identified in Brazil. (Yang *et al.*, 2018; Di Mento *et al.*, 2018; Garza-Ramos *et al.*, 2018) [61, 62, 63]. GNB resistance to colistin was prevalent in this study, which may represent the continued existence of colistin resistance in the region of study. This trend could be linked to the patient's being exposed to sublethal dosages of colistin as a last-line medication in the treatment of recurring or severe GNB infections.

ESBL and Carbapenem biofilm forming strain demonstrate resistance to ciprofloxacin, amikacin, imipenem, ofloxacin amoxicillin/clavulanate, nalidixic acid, azetronam and tetracycline which are antibiotic for the treatment of complicated GNB infections. The *in vitro* reduce susceptibility of this antimicrobial agent, make their futuristic use uncertain due to the complexity of resistant arsenal unleashed by the GNB to truncate the drug mode of action. The implication of this may result in the emergence of Difficult-To- Treat (DTT) GNB infection.

The high prevalence of resistance GNB represented serious resistant patterns due to biofilm formation. In biofilms, the

EPS that leads to the adaptive responses to stress, low antibiotics penetration, and the formation of per sister cells are hypothesized to constitute a multilayered defense, increasing the difficulty of eradication, especially when combined with the bacteria's resistance. As antibiotic resistance and the ability of bacteria to build biofilm are crucial factors in the global spread of bacteria, there is need to clearly ascertain and elaborate the relationship between these factors.

## Conclusion

This study shows that majority of the biofilm producing GNB displayed resistant phenotypes. This resistant determinants were related to the multi-drug resistance phenotype to different classes of antimicrobial agent. The prescription of antibiotics in our area of study may continue to become a formidable challenge as most of the last-line antibiotic such as imipenem, colistin, amikacin, β-lactamase inhibitors showed reduce susceptibility. This shows that a single antimicrobial regimen is insufficient to eradicate biofilm-forming pathogens. As a result, controlling infections with currently available antibiotics and assessing the results have become critical and urgent measures for the successful treatment of biofilm-associated infections.

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