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Evaluation of Co-production of Colistin Resistance and ESBL Genes among Gram-negative Clinical Isolates from Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria

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Abstract

The emergence of antimicrobial resistance (AMR) is a major threat to global health. Its effects include high mortality and morbidity rates, treatment failure, and increased treatment costs. This study aimed to evaluate the co-production of colistin-resistant and extended-spectrum beta-lactamase (ESBL) genes among Gram-negative clinical isolates from Usmanu Danfodiyo University Teaching Hospital in Sokoto. Gram-negative bacteria were isolated from clinical specimens, including urine, feces, and wound aspirates. The Double-Disk Synergy Test and the Colistin Agar Test, respectively, were used to phenotypically validate the existence of colistin resistance and ESBL. Polymerase chain reaction (PCR) was used for molecular characterization. Primers were used to target genes linked to colistin resistance (mcr-1 and mcr-2) and ESBL genes (blaCTX-M, CTX-M 1, CTX-M 2, and CTX-M 8). The findings indicated that 13.9% of the isolates displayed co-production of Colistin and ESBL, and of these isolates, 60% had blaCTX-M genes, and 20% had CTX-M 8 linked to ESBL production. However, the presence of colistin resistance genes was not detected by PCR. Therefore, molecular analysis did not confirm the existence of the colistin resistance genes (mcr-1 and mcr-2) in these isolates. Consequently, the findings showed no molecular co-production of the ESBL and colistin resistance genes. This work emphasizes how crucial it is to look into molecular and phenotypic traits to completely comprehend how colistin resistance and ESBL genes coexist in Gram-negative isolates. More research is required to investigate other mechanisms behind the resistance phenotypes identified.

Keywords: Colistin resistance, ESBL genes, Co-occurrence resistance, Gram-negative isolates.

INTRODUCTION

According to Aslam *et al*. (2018), antimicrobial resistance (AMR) is a major worldwide danger to the health of people, animals, and the environment. This is because "superbugs," or drug-resistant bacteria, have become more prevalent and persistent (Davies and Davies 2010). AMR is a major danger to public health worldwide due to the ongoing rise in antibiotic consumption worldwide (Van Boeckel *et al*., 2014; Laxminarayan *et al*., 2016). The World Health Organization (WHO) estimates that AMR causes 700,000 deaths worldwide annually, and if the current trend of rising AMR is not stopped, 10 million people could die each year from resistant bacterial infections by the year 2050 (Kupferschmidt, 2016; O'Neill, 2014).

The emergence of resistance in bacterial pathogens, such as nontyphoidal Salmonella, *Staphylococcus aureus, Klebsiella pneumoniae*, and *Escherichia coli*, which cause the majority of nosocomial infections and community-acquired infections globally, is a particular cause for concern (WHO, 2014). The World Health Organization is particularly concerned about the emergence and spread of resistance to antibacterial drugs frequently used to treat infections caused by common bacterial pathogens. Examples of these pathogens include *E. coli* resistance to third-generation cephalosporins and fluoroquinolones, *K. pneumoniae* resistance to third-generation cephalosporins and carbapenems, *S. aureus* resistance to methicillin, *S. pneumoniae* resistance to penicillin, and nontyphoidal Salmonella resistance to fluoroquinolones (WHO, 2014).

Multidrug resistance (MDR) has increased and is now a major global source of morbidity and mortality (Dadgostar, 2019). Antibiotic therapy for bacterial infections may become more limited in the near future due to resistance to antibiotics used as a last resort, such as colistin and newer-generation cephalosporins (Fair & Tor, 2014). Colistin and polymyxin B have been used for preventive and therapeutic purposes in poultry production (Sun *et al*., 2018).

Colistin is a last-resort antibiotic for treating infections caused by Multidrug-resistant Gram-negative bacteria (Sharma *et al.*, 2022). However, its effective use is seriously threatened by the emergence of resistance mediated by mobile colistin-resistant (mcr) genes (Anyanwu *et al.*, 2021). Even though the clinical significance of the ESBL and mcr genes is well established, there is a knowledge gap in the prevalence and distribution of mcr genes among colistin and ESBL in low-income countries such as Nigeria (Zhang *et al.*, 2021). Compared to infections produced by non-ESBL-producing bacteria of the same species, Rozenkiewicz et al. (2021) report that ESBL-producing bacteria can increase mortality, length of hospital stays, and healthcare expenses.

Regrettably, colistin-resistant infections have spread throughout the world as a result of the overuse and abuse of colistin in human and veterinary medicine. However, the emergence of bacteria resistant to colistin may also happen without prior colistin exposure, leaving medical professionals ill-prepared to treat patients (Mlynarcik & Kolar, 2019). Because they are sometimes the only antimicrobial drug active against multidrug-resistant Gram-negative bacteria (MDR GNB), polymyxins are now mostly effective against life-threatening Gram-negative infections (Kaye *et al.*, 2016). Numerous investigations revealed a sharp rise in the frequency of colistin resistance in Enterobacteriaceae. Because MDR bacteria can evolve colistin resistance through mutation or adaption processes, clinicians need to be on the lookout for this phenomenon. Colistin should only be prescribed as a last option for treating infections, according to the scientific community, specialists, government authorities, and public-private consortiums, who have called for a decrease in the drug's use (Sharma *et al.*, 2022).

The beta-lactamases known as Extended Spectrum Beta-lactamases (ESBLs) provide resistance against oxyimino "second- and third-generation" cephalosporins, such as aztreonam and cefotaxime (CTX), ceftriaxone, cefpodoxime, and ceftazidime (Moawad *et al.*, 2018). Soon after the third-generation cephalosporins, CTX and ceftiofur (CEF), were introduced into clinical settings in 1980, there were reports of ESBL-producing bacteria (Yamasaki *et al.*, 2017). Around 350 ESBL genes have been identified; these genes are primarily produced by point mutations of the classical SHV-1 and TEM-1 Beta-lactamases, with the CTX-M kinds becoming more and more dominant (Brolund & Sandegren, 2016). Over the past ten years, animal-origin E. coli isolates that produce ESBL have made blaCTX-M-55, one of the CTX-M enzymes, the most common kind (Abbas *et al.*, 2019). However, among isolates of human origin, blaCTX-M-15 appears to be the most prevalent type (Woerther *et al.*, 2013).

Additionally, ESBLs are beta-lactamase enzymes that can hydrolyze ß-lactam antibiotics, such as Penicillins, Aztreonam, and first to fourth-generation Cephalosporins, excluding Cephamycins, Moxalactam, and Carbapenems, (Rajivgandhi *et al.*, 2021). However, ß-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam suppress the activity of beta-lactamase enzymes. The most worrisome thing is that, in addition to ß-lactams, ESBL production can also lead to resistance to other antibiotics like aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole, and chloramphenicol (Taghizadeh *et al.*, 2018).

Globally, uropathogens that produce ESBLs are becoming an issue (Mahmoud *et al.*, 2020). Consequently, there are significant therapeutic ramifications from co-producing ESBL resistance genes and colistin. Treatment options are severely limited by co-producing isolates, which frequently show resistance to numerous types of antibiotics. This increases the likelihood of treatment failure and unfavorable patient outcomes, posing a serious challenge in managing infections brought on by these strains. Also, understanding the underlying genetic mechanisms of colistin resistance and its spread requires the molecular characterization of colistin and ESBL-resistant genes (Cao *et al.*, 2018). To improve infection control procedures, antimicrobial stewardship, and treatment approaches, it is important to look at the frequency, genetic diversity, and possible transmission pathways of mcr genes in colistin-resistant isolates (Shen *et al.*, 2018). Thus, the need to carry out this research to evaluate the co-production of Colistin resistance and ESBL genes among Gram-negative clinical isolates from Usmanu Danfodiyo University Teaching Hospital, Sokoto, to enhance management and control of drug-resistant infections at the center with the understanding of the incidence and genetic properties of mcr and ESBL genes in Gram-negative isolates.

MATERIALS AND METHODS

Methods

Source of Test Bacteria Isolates

Gram-negative isolates were obtained from the Medical Microbiology Laboratory, Usmanu Danfodiyo University Teaching Hospital (UDUTH) in Sokoto, Nigeria. The bacteria stock was transferred to the Pharmaceutical Microbiology Laboratory, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria, for further analysis.

Antibiotic Susceptibility Testing (AST)

AST was carried out using Kirby-Bauer's disk diffusion method. Fifty (50) Mueller Hinton agar plates were prepared by suspending 38g of Mueller Hinton agar in 1000ml of distilled water, then boiled on a hot plate until it dissolved completely, after which it was sterilized in an autoclave at 121°C for 15 minutes. The agar mixture was allowed to cool, after which approximately 20ml each was poured into fifty (50) sterile Petri dishes using an aseptic technique and then left to solidify at room temperature.

Fifty (50) inoculums were prepared using a sterile loop to transfer 2 to 3 isolated colonies of the organism from each quadrant of the subcultured agar plates to test the tubes containing sterile saline. The turbidity was adjusted to match a 0.5 McFarland standard. The agar plates were then inoculated by dipping a sterile swab stick into the broth, and excess moisture was expelled by pressing the swab against the side of the tube. The surface of the agar was then swabbed completely, ensuring that no areas were left un-swabbed. It was then turned 90 degrees, and the swabbing process was repeated, after which the swab stick was run around the plate's circumference before discarding it in the discard bag. This was done for each of the isolates.

The surfaces were then allowed to dry for about 5 minutes, after which a pair of sterile forceps were used to remove the combined antibiotic disk from the dispenser and placed on the agar ensuring it maintained good contact with the agar surfaces. The multi-disk antibiotic containing Amoxicillin Clavulanate (30µg), Cefotaxime (25µg), Imipenem/cilastatin (10/10µg), Nitrofurantoin (30µg), Cefuroxime (30µg), Ceftriaxone sulbactam (45µg), Ofloxacin (5µg), Gentamycin (10µg), Nalidixic acid (30µg), Ampiclox (10µg), Cefixime (5µg), and Levofloxacin (5µg)) was then placed at the surface of the inoculated agar plates. The plates were allowed for a pre-diffusion time of 15 minutes and then incubated upside down at 37°C for 18 – 24 hours. The inhibition zones were measured and interpreted according to CLSI (2021) standards.

Screening and confirmatory Tests for ESBL Production

The screening test was conducted similarly to the antimicrobial susceptibility test, as described. Disks containing the third-generation cephalosporins ceftazidime (30µg) and cefotaxime (30µg) were used for the screening process, unlike AST.

The double disc synergy test (DDST) was used for clinical samples that passed the screening test to confirm the ESBL phenotype. Amoxicillin/clavulanic acid (20/10µg) and two third-generation cephalosporins, ceftazidime (30µg) and cefotaxime (30µg) were used. Ceftazidime and cefotaxime were added to Mueller Hinton Agar with a sterile needle and positioned 15 mm apart from the center of amoxicillin/clavulanic acid (CLSI, 2021). Following a 24-hour incubation period at 37°C, the plates were inspected for the development of a zone of inhibition extended toward the amoxicillin/clavulanic acid. The development of ESBL is indicated by an enhancement of the zone of inhibition of one or both ceftazidime and cefotaxime towards the amoxicillin/clavulanic acid disc (CLSI, 2021).

Phenotypic Detection of Colistin Resistance

The colistin agar dilution method was used for phenotypic detection of colistin resistance. The CLSI (2021) procedure was adopted at 3µg/ml of colistin sulfate. Using a poison balance, a 3 mg powder of colistin sulfate was aseptically weighed and then added to 500 milliliters of distilled water. After suspending 38g of Mueller Hinton agar powder in 500ml of distilled water and boiling it on a hot plate until it dissolved entirely, the mixture was autoclaved for 15 minutes at 121°C before being allowed to cool. After carefully mixing the colistin sulfate stock solution into the agar mixture, 20 milliliters of each was aseptically poured into sterile Petri dishes and allowed to harden at room temperature. Spots measuring about 20 mm in diameter were created on the agar surface using sterile swab sticks dipped into bacterial inoculums that had been prepared and adjusted to the 0.5 McFarland standard. After 18 to 20 hours of upside-down incubation at 35°C, the growth of one or more colonies was regarded as resistant.

Molecular Characterization

DNA Extraction

Table 1: Primer sequences of mcr and CTX-M

Primers	Nucleotide sequence (5'-3')	Size (bp)
mcr-1F	CGGTCAGTCCGTTTGTTC	309
mcr-1R	CTTGGTCGGTCTGTAGGG	
mcr-2F	TGGTACAGCCCCTTTATT	1,747
mcr-2R	GCTTGAGATTGGGTTATG	
BlaCTX-M-F	TGCGATGTGCAGTACCAG	
BlaCTX-M-R	ATCGTTGGTGGTGCCATA	550
BlaCTX-M-1-F	GGACGTACAGCACTTGC	
BlaCTX-M-1-R	CGGTTCGCTTTCACCTT	891
BlaCTX-M-2-F	GTGCTTAAACAGAGCGAG	
BlaCTX-M-2-R	CCATGAATAAGCAGCTGA	624
BLaCTXM-8-F	ACGCTCAACACCGCGATC	
BlaCTX-M-8-R	CGTGGGTTCTCGGGGATA	480

Polymerase Chain Reaction (PCR)

Multiplex PCR was utilized to amplify the DNA. In an Eppendorf tube, 90µl of nuclease-free water and 10µl of the previously reconstituted primers (Table 1) were combined to create working primers. Next, 100 µl of a master mix, 5µl of each working primer, and 60µl of nuclease-free water were combined to create the cocktail. Ultimately, 18µl of the cocktail and 2µl of each extracted DNA were combined to create a final volume of 20µl, which was used to prepare the PCR reaction mixes. "At 94 °C: initial denaturation for 5 min and 25 cycles of denaturation for 30 seconds, then annealing at 52 °C for 30 s, and finally at 72 °C: extension for 30 s and a final extension for 5 min" was the protocol followed when it was transported into the thermocycler for amplification. In order to evaluate the amplicons, agarose gel electrophoresis was used.

Agarose Gel Electrophoresis

A 1.5g agarose powder was weighed, dissolved in 100 ml of Tris base, acetic acid, and EDTA (TAE) buffer, and then put into the water bath for full dissolution after ensuring the electronic digital balance was calibrated. After letting the melted agar cool to around 50°C, 5µl of ethidium bromide was added and mixed with a swirl. After the gel was put into an agarose gel tank and 500 milliliters of 0.5x TBE buffer were added, it was left for 30 minutes to solidify in the broad Bio-rad gel casting device. Subsequently, a 6x loading dye was combined with 10µl of the standard and added to each designated well. The electrophoresis power pack was set to operate at 10 volts for 30 minutes after the tank was closed, and the Biorad gel imager was used to view the gel's results. The outcome was determined by referring to the forward and reverse base pair base pairs.

RESULTS

The percentage distribution of the isolates' sources and the identified bacteria prevalence

Of the fifty isolates that were obtained, 45 (90.0%) were from urine samples, 03 (6.0%) from wound swabs, and 02 (4.0%) from stool cultures as shown in Fig. 1. In the meantime, *Escherichia coli* 39 (78%) was the most isolated Gram-negative pathogen, followed by *Pseudomonas spp*. 10 (20%) and only one isolate (2%) of *Klebsiella spp*., according to the frequency and distribution of different bacterial isolates as shown in Fig. 2.

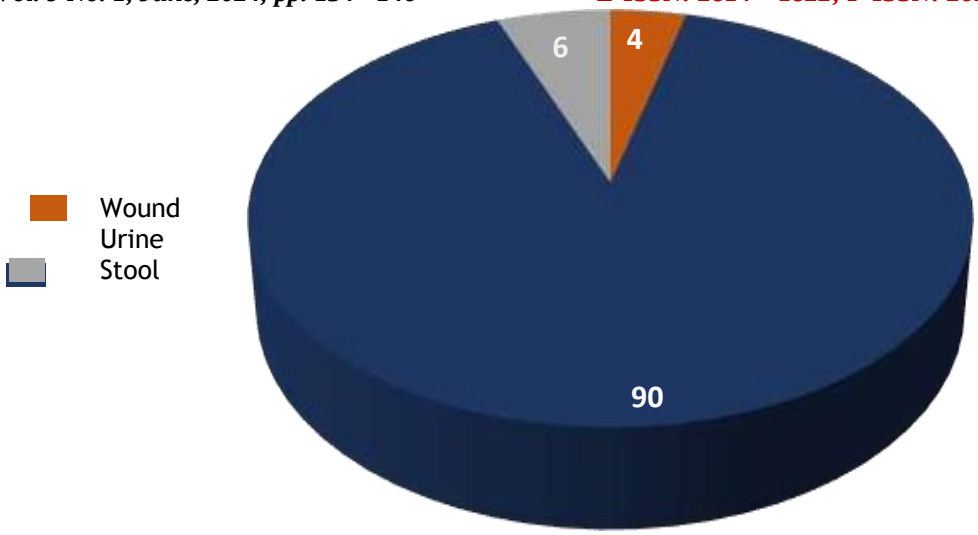


Fig 1: Distribution of the sources of Isolates

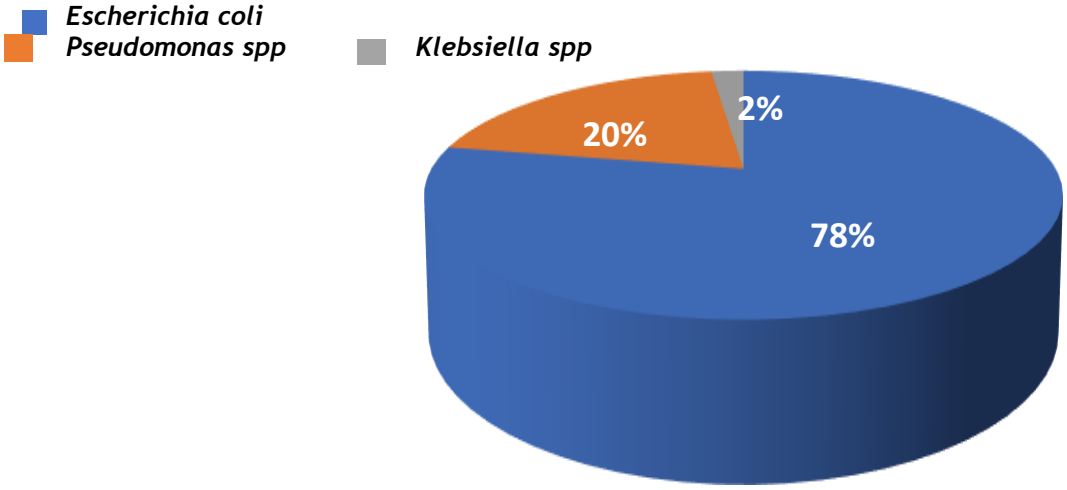
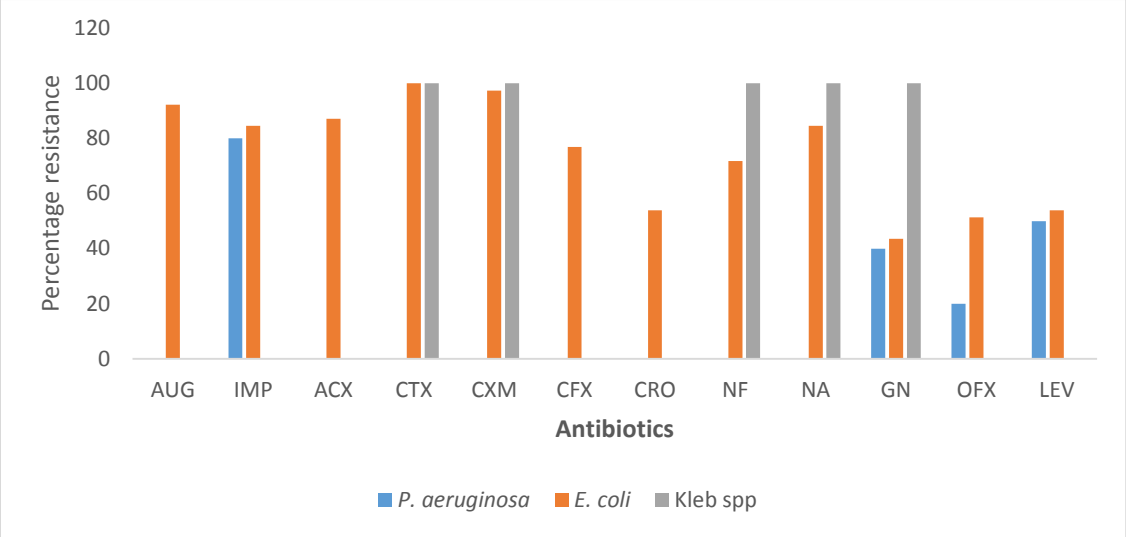


Fig 2: Occurrence of the identified Organisms

Antibiotic Susceptibility Test (AST)

Gram-negative bacterial isolates have varying degrees of antibiotic resistance, as demonstrated by the antibiotic susceptibility test (AST) shown in Figure 3. Remarkably, *Klebsiella species* showed complete resistance to gentamicin, nalidixic acid, and cephalosporin. *E. coli* showed varying degrees of resistance, ranging from 100% to 78% to tested ß-lactam antibiotics in Augmentin. Even so, resistance to the remaining antibiotics was least noticeable regarding gentamicin (44%) and the tested fluoroquinolones (54%). Compared to gentamicin, which only showed 40% resistance, imipenem, a ß-lactam, revealed an 80% resistance in Pseudomonas species. Concurrently, ceftriaxone plus sulbactam showed increased cephalosporin sensitivity; this was explained by the possibility that sulbactam inhibits beta-lactamase. Compared to other antibiotics, cephalosporins with beta-lactamase inhibitors, like ceftriaxone with sulbactam and fluoroquinolones, showed greater susceptibility.



AUG = AUGMENTIN, CTX = CEFOTAXIME, IMP = IMIPENEM, NF = NITROFURANTOIN, CXM = CEFUROXIME, CRO = CEFTRIAXONE/SULBACTAM, OFX = OFLOXACIN, GN = GENTAMYCIN, NA = NALIDIXIC ACID, ACX = AMPICLOX, CFX = CEFIXIME, LEV = LEVOFLOXACIN

Fig. 3: Resistant profiles of the isolates from different sources

Screening for ESBLs Producers

Of the 30 isolates, 38.8% (14) were found to be resistant to both cephalosporins and another 77.7% (n=28) to only cefotaxime, while 44.4% (n=16) isolates show resistance to only ceftatxidime. The screening test revealed that 83.3% (30) were resistant to at least one of the 2 third generation cephalosporins and are potential ESBL producers (Fig. 4).

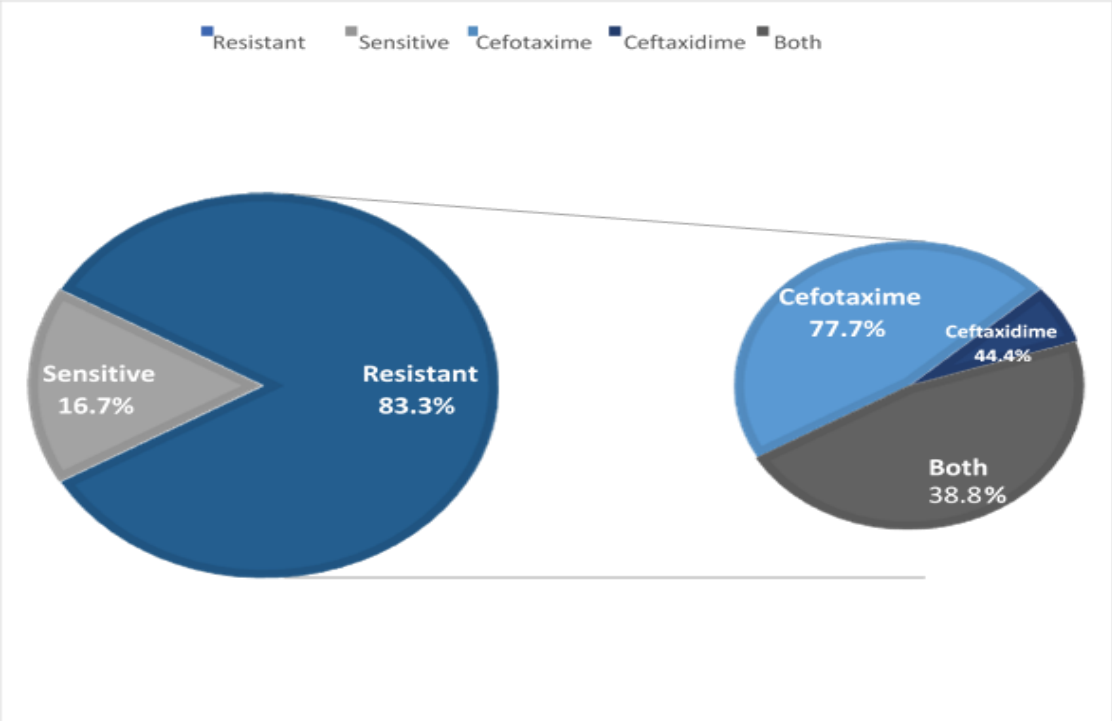


Fig. 4: Assessment of suspected ESBL producers

Phenotypic Detection of ESBL Producers

According to the phenotypic test, 23.3% (n=7) of the suspected ESBL producers had negative phenotypic results, whereas 76.7% (n=23) of the suspected ESBL producers were found to be positive for ESBL production (Fig. 5). 23 Gram-negative bacterial isolates were shown to be ESBL producers phenotypically out of all the isolated organisms (Fig. 5).

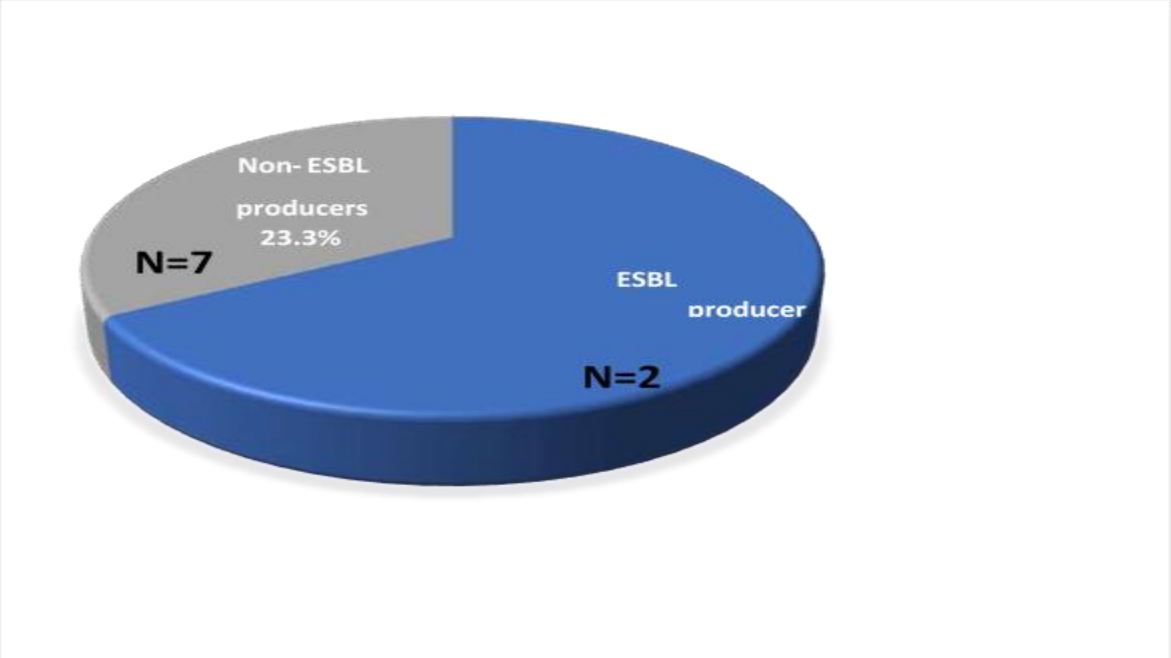


Fig. 5: Assessment of suspected ESBL producers for ESBL production

Phenotypic Detection of Colistin Resistance
As illustrated in Fig. 6, of the 36 isolates, 22.2% (n=8) were resistant to colistin. *P. aeruginosa* did not exhibit any resistance, and all resistant isolates (100%) were *E. coli*.

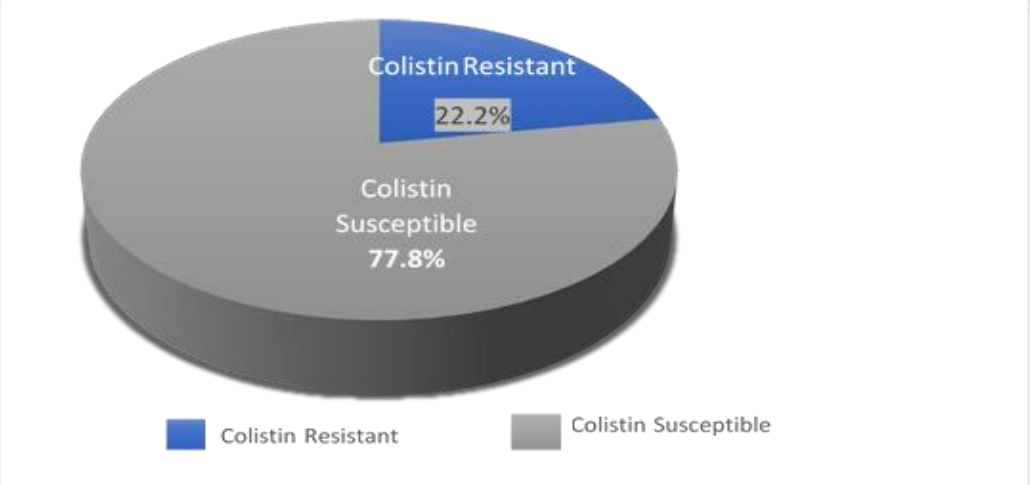


Fig. 6: Phenotypic result for colistin resistance

Evaluation of clinical isolates for phenotypical co-production of colistin and ESBL resistance
Based on the colistin and ESBL phenotypic data, 5.9% of the isolates coproduced colistin and ESBL resistance, whereas 86.1% (31) did not coproduce. (Figure 7).

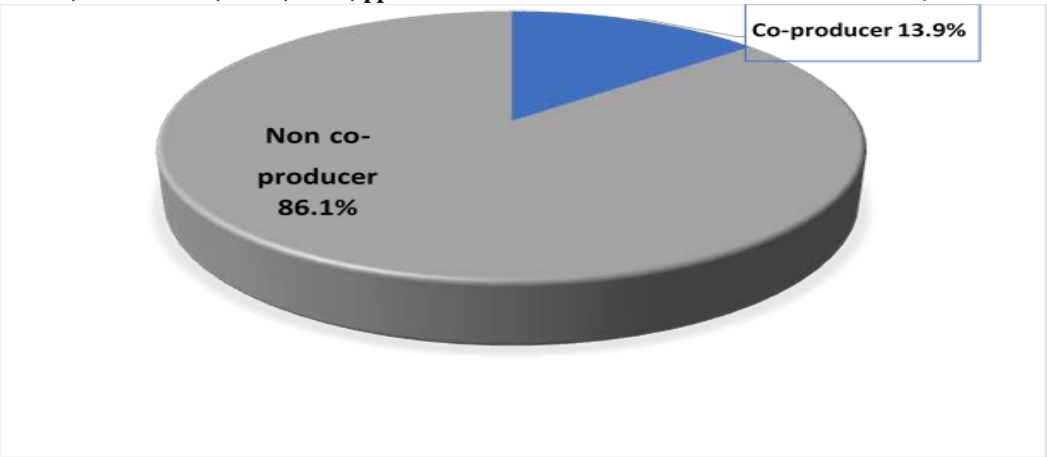


Fig. 7: Percentage of phenotypic co-producers

Gel image of PCR product for CTX-M

As shown in Fig. 8, four of the five isolates, or eighty percent (80%), have ESBL genes (three CTX-M at 550 bp and one CTX-M 8 at 490 bp).

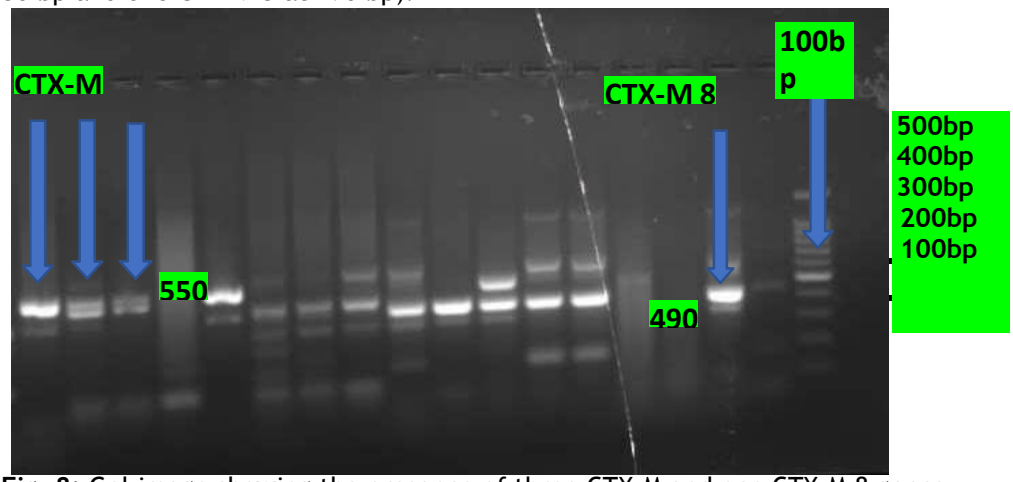


Fig. 8: Gel image showing the presence of three CTX-M and one CTX-M 8 genes

Gel image of PCR product for *mcr*

Amplification bands (500 bp) were obtained from only three of the five isolates (60%), and these bands did not match the predicted sizes for the mcr-1 (307) and mcr-2 (1747) genes, respectively (Fig. 9).

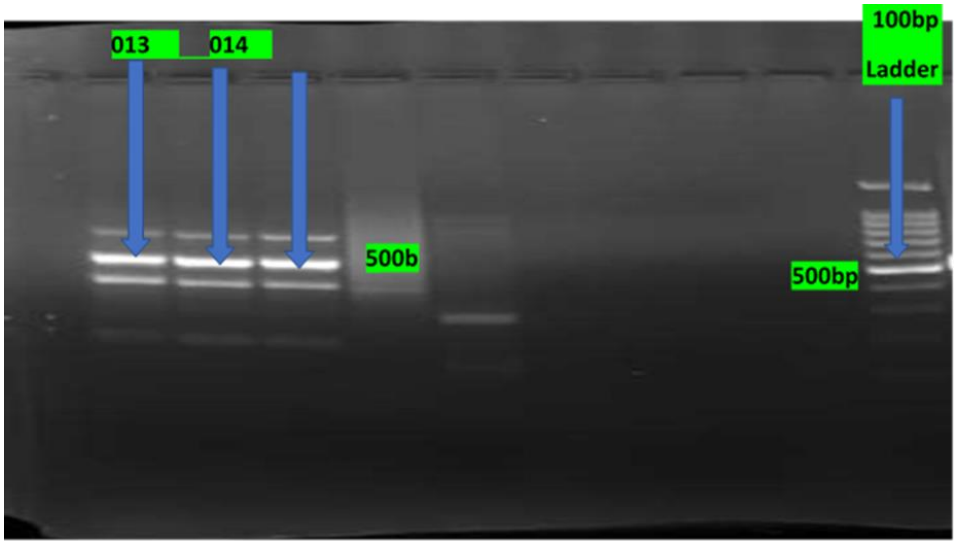


Fig. 9: Gel image of colistin-resistant DNA showing bands at 500bp

DISCUSSION

A high occurrence of resistance was noted based on the results at different points in the investigation. According to Abbas *et al.* (2019), the majority of Gram-negative bacteria pathogens in this investigation were *E. coli* (78%), which is consistent with several earlier results from Nigeria, including those from Sokoto state (Nuhu *et al.*, 2015, Tanko *et al.*, 2020). The antibiotic susceptibility test (AST) results indicated that resistance to aminoglycoside, cephalosporins, fluoroquinolones, and penicillin was highly prevalent. This resistance pattern is in line with findings from a number of studies, including those by Omoya and Ajayi (2016), Nyandwi *et al.* (2017), and Udoh *et al.* (2020), which reported high resistance rates to Augmentin, cephalosporin, and fluoroquinolones among bacterial isolates, particularly *E. coli* and *Klebsiella species*. It is also compatible with research conducted in Sokoto, Northeastern Nigeria, by Adeluola *et al.* (2018) and Olowo-Okere *et al.* (2018), as well as Nuhu *et al.* (2015).

According to Mohammed *et al.* (2016), Ugwu *et al.* (2020), and Olufunke *et al.* (2014), there was a high prevalence of ESBL producers (76.7%) in this study as well, which is in contrast to research done in Kano, Northwestern Nigeria, where ESBL prevalence was 9.25%, 27.7% in Southeastern Nigeria, and 26.1% in Southwestern Nigeria. This is, however, in line with studies by Peculiar-Onyekere *et al.* (2019) in Nnewi, Nigeria, which revealed a high frequency of 67.7% for ESBL-mediated UTIs in pregnant women.

Additionally, among ESBL-producing Enterobacteriaceae isolates, CTX-M-2, CTX-M-8, and CTX-M-9 groups were revealed to be the most often discovered CTX-M-type enzymes (Chagas *et al.*, 2011). This is consistent with the study, which found that the most common subtypes were CTX-M-2 and CTX-M-8. However, the CTX-M, CTX-M-1, CTX-M-2, and CTX-M-8 subtypes are the only ones we address in this study. Additionally, phenotypic results for colistin revealed that 22% of the isolates were resistant to the drug. Vounba *et al.* (2019), Arif *et al.* (2022), and Ngbede *et al.* (2020) reported similar results in Vietnam, where *P. aeruginosa* had a 20% resistance rate to colistin, 22.8% in Pakistan among Gram-negative uropathogens, and 17.0% in Nigeria. This, however, differs from a study by Li *et al.* (2023) that was carried out in China and found a prevalence of 53.47%.

Furthermore, a study conducted in Oyo State, Nigeria, discovered that 84% of clinical isolates of *E. coli* and Klebsiella spp. Obtained from blood samples of outpatients and inpatients were colistin-resistant (Hassen *et al.*, 2022), whereas 43.6% of resistance was reported from Lagos (Egwuatu *et al.*, 2021). It was observed phenotypically that there was colistin and ESBL resistance co-production, as 13.9% (N=5) of the isolate were co-producers. Nevertheless, there was no co-production at the molecular level after molecular characterization. Only three of the five isolates produced 500 bp amplification bands for colistin, and those bands did not match the predicted sizes for the mcr-1 (307) and mcr-2 (1747) genes. Four of the five isolates (80%) displayed the presence of ESBL genes (three CTX-M and one CTX-M 8). The differences that have been found show that the bacterial community is genetically diverse. It's possible that the primers' targets, the mcr-1, mcr-2, and CTX-M genes, are absent from the two isolates lacking bands in colistin and one in ESBL. There could be other resistance genes or genetic variations in these isolates that were missed by the particular primers used. Resistance can develop through a variety of mechanisms. Reports of isolates resistant to colistin but lacking the mcr genes have surfaced. For instance, colistin-resistant Enterobacteriaceae isolates from several sources were examined in a study by Olaitan *et al.* (2014). Several isolates lacked the known mcr genes yet exhibited phenotypic resistance to colistin. Rather, whole-genome sequencing identified mutations in genes related to the manufacture or modification of LPS, resulting in changed outer membrane characteristics and decreased susceptibility to colistin.

Cannatelli *et al.* (2013) found isolates of *Klebsiella pneumoniae* resistant to colistin and did not have the mcr-1 gene in another investigation. These isolates' genome sequencing revealed gene changes linked to the two-component regulatory system pmrAB, which changed LPS and boosted colistin resistance (Olowo-Okere & Yacouba, 2020). These instances demonstrate the presence of mechanisms for colistin resistance that go beyond the identified mcr genes. When examining isolates that show phenotypic resistance but lack the mcr genes, it is crucial to consider other resistance mechanisms. When the primers used in the PCR reaction bind to unwanted DNA sequences, non-specific amplification may result. Larger bands that might not be connected to the important colistin resistance genes may be produced due to the amplification of non-target areas of the genome. High primer concentrations, poor primer design, or non-specific primer binding due to sequence similarities with other genome parts can all contribute to non-specific amplification.

Contamination during the PCR process may introduce extraneous DNA templates, which could lead to the formation of unexpected bands. A number of things, including reagents, equipment, and past PCR reactions, can contaminate an area. The bands that were seen might have been caused by contamination with 500 base pairs or fewer DNA fragments.

Additionally, research has shown that colistin resistance genes, such as mcr-1 and mcr-2, exhibit genetic diversity. For instance, Cannatelli *et al*. (2013) discovered an allelic variation of the PmrB sensor kinase in an *Escherichia coli* strain that causes colistin resistance. When employing particular primers, this genetic diversity can lead to varied amplicon sizes, particularly if insertions or deletions are in the target area. Olaitan *et al*. (2016) found that one isolate of *Escherichia coli* carried the mcr-1.2 gene variant, which is a variation of the mcr-1 gene. A 484-base pair insertion resulted in the mcr-1.2 gene having a greater amplicon size than the original mcr-1 gene. This demonstrates how different gene variants can exist within the same family of colistin-resistance genes. Numerous researches have also reported on gene rearrangements or insertions involving colistin resistance genes.

For example, Olaitan *et al*. (2014) found that the inactivation of the PhoP/PhoQ regulator mgrB led to the global establishment of colistin resistance in *Klebsiella pneumoniae*. Genetic insertions and rearrangements involving mgrB were identified as the cause of the inactivation. When PCR primers target certain genetic events, the amplicon sizes may increase. In a study published in, Borowiak *et al*. 2017 found that isolates of *Escherichia coli* carried a novel colistin resistance gene called mcr-3.3. Compared to the mcr genes that were previously reported, such as mcr-1 and mcr-2, the amplicon size of the mcr-3.3 gene was greater. This discovery indicates more colistin resistance genes with various amplicon sizes. Therefore, the bigger bands seen in these isolates may also point to the existence of novel, as yet uncharacterized, colistin resistance genes. These genes have extra genetic elements or mutations that lead to a bigger amplicon size, yet they may have some sequence similarity with mcr-1 or mcr-2. It would take more research to locate and describe these new genes, such as whole-genome sequencing or gene-specific PCR.

In another study, Xavier et al. (2016) looked at colistin-resistant Escherichia coli isolates that exhibited resistance traits but lacked the mcr-1 or mcr-2 genes. After more investigation, they discovered mcr-3, a novel plasmid-mediated colistin resistance gene that shared tight ties with mcr-1. This indicates that there are more colistin-resistance genes that traditional PCR techniques might overlook.

It's possible that the bacterial strains under study separately acquired the genes causing ESBL and colistin resistance. This implies that diverse processes, such as horizontal gene transfer from several sources or mutations happening in various genetic locations, may have contributed to the acquisition of the resistance genes. According to this hypothesis, colistin and ESBL resistance do not directly result from co-production; their co-occurrence is accidental.

In the absence of co-production, a number of investigations have documented the presence of ESBL resistance genes and colistin. For instance, a study by Smith et al. (2017) examined the genetic profiles of Enterobacteriaceae that produced ESBL and discovered that certain strains had colistin resistance and ESBL genes, but the genes were not co-produced or physically connected. The lack of co-production at the molecular level may also be explained by genetic variations between the strains or populations that are being studied. The co-occurrence or co-expression of these resistance genes may not be encouraged by the genetic backgrounds of the ESBL- and colistin-resistant bacteria. Variations in the genetic background, regulatory components, environmental factors, or other factors influencing the expression of these resistance determinants may cause this. The coexistence of colistin and carbapenem resistance in clinical isolates of *Klebsiella pneumoniae* was examined in a study by Liakopoulos *et al*. (2016). They discovered that although there was evidence of both resistance mechanisms, co-production was not evident. According to the researchers, the lack of co-production was likely driven by variations in the strains' genetic backgrounds. It is feasible for isolates to show phenotypic co-production resistance to both ESBL and colistin without disclosing the known colistin resistance genes, according to the literature that is currently available. Further research must determine whether the 500 base pair bands are real or PCR artifacts. It is also important to consider the potential that novel colistin resistance genes or gene variants/subtypes could explain the bigger bands seen in this study.

More research is required to discover and describe these genes and their possible involvement in colistin resistance, such as functional studies and genetic sequencing. These findings further demonstrate the intricacy and diversity of colistin resistance pathways and their possible implications for clinical practice and public health.

CONCLUSION

The study reveals high-level resistance to fluoroquinolones, cephalosporins, aminoglycosides, and penicillin by Gram-negative bacteria isolates from Usmanu Danfodiyo University Teaching Hospital, Sokoto, and a high percentage (76.7%) of the isolates produced ESBL and had several CTX-M genes (CTX-M and CTX-8). The study detected colistin and ESBL co-production phenotypically; however, PCR did not detect co-production. These results imply that novel or alternate co-existing resistance mechanisms facilitate the isolates' co-production of resistance.

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