Interdisciplinary Research in Medical Sciences Specialty
ISSN: 3062-4401
2023, Volume 3, Issue 2, Page No: 17-24
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Available online at: www.galaxypub.co/page/journals

Galaxy Publication

Prevalence of AmpC and Extended-Spectrum Beta-Lactamase-Producing Bacteria in Livestock and Poultry Environments in Southeast Nigeria

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Received: 14 May 2023; **Revised:** 27 August 2023; **Accepted:** 29 August 2023

ABSTRACT

Antimicrobial resistance (AMR) occurs when microorganisms no longer respond to the therapeutic effects of antibiotics. Extended-spectrum beta-lactamase (ESBL) and AmpC enzymes are key factors in AMR, diminishing the effectiveness of essential antibiotics. This study investigated the detection and antimicrobial susceptibility of ESBL- and AmpC-producing bacteria found in environments associated with livestock and poultry. A combination of bacteriological and molecular methods was used for bacterial isolation and identification. Disk diffusion and PCR were used to confirm ESBL and AmpC production. ESBL-producing bacteria were detected in poultry samples at rates of 4%, 1%, and 2% in E. coli, Klebsiella species, and P. aeruginosa, respectively. In livestock samples, ESBL production was observed in E. coli (5%), Klebsiella species (2%), and P. aeruginosa (4%). AmpC-producing bacteria were found in E. coli (3%), Klebsiella species (2%), and P. aeruginosa (1%) in poultry environments, while in livestock environments, AmpC was detected in E. coli (7%), Klebsiella species (3%), and P. aeruginosa (6%). Both ESBL- and AmpC-positive strains showed a significant reduction in susceptibility to carbapenems and cephalosporins. PCR analysis revealed the presence of CTX-M-15 genes in 20% and FOX-1 genes in 25% of the bacteria, which are responsible for mediating resistance to ESBL and AmpC. These findings identify the key genetic factors contributing to bacterial resistance in Southeast Nigeria and emphasize the importance of continuous monitoring and surveillance to limit the spread of AMR, as it poses a significant risk to effective antibiotic treatments.

Keywords: ESBL, AmpC enzymes, Livestock, Multidrug-resistant bacteria, Poultry

How to Cite This Article: Ejikeugwu C, Obum-Nnadi C, Onu E, Adonu C, Ujam N, Iroha C, et al. Prevalence of AmpC and Extended-Spectrum Beta-Lactamase-Producing Bacteria in Livestock and Poultry Environments in Southeast Nigeria. Interdiscip Res Med Sci Spec. 2023;3(2):17-24. https://doi.org/10.51847/i2JznwNOSC

Introduction

Antimicrobial resistance (AMR) represents a major global health threat, particularly affecting developing healthcare systems. It involves the development, spread, and exchange of antibiotic resistance genes (ARGs) and resistant bacteria across animals, humans, and the environment, significantly impairing the effectiveness of treatments for common bacterial infections. Among the most clinically significant contributors to AMR-related fatalities are members of the Enterobacteriaceae family and non-enteric bacteria. These bacteria are responsible

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for a high proportion of AMR deaths, both in food production systems and healthcare settings in countries at various income levels. AMR can spread through mobile genetic elements (MGEs) such as plasmids, which can transfer resistance traits through mechanisms like conjugation between bacteria in the same environment. The presence of AMR bacteria promotes the accumulation of MGEs, fostering the development and propagation of ARGs. It has been projected that by 2050, AMR will be responsible for 10 million deaths annually worldwide [1]. The indiscriminate use of antibiotics, especially in livestock and poultry farming, plays a significant role in the emergence and transmission of AMR/ARGs within both community and hospital environments. Extended-spectrum beta-lactamases (ESBLs) and AmpC enzymes are key resistance mechanisms that enable bacteria to evade the effects of certain antibiotics. The rising occurrence of these resistant bacteria and associated ARGs poses a substantial public health challenge, as AMR limits the available therapeutic options for bacterial infections [2-6]. ESBLs and AmpC enzymes provide bacterial pathogens, including *P. aeruginosa*, *E. coli*, and *Klebsiella* species, with the ability to resist the action of second- and third-generation cephalosporins, crucial antibiotics used to treat a wide range of bacterial infections [5-7]. This resistance makes effective antimicrobial therapy difficult and complicates the management and prognosis of infections caused by these pathogens, which are among the leading resistant microorganisms in both community and hospital settings worldwide [7-9].

The global spread of antimicrobial resistance (AMR) is a significant public health threat, particularly in hospitals, where it complicates the process of selecting effective antibiotics for treating infections caused by resistant pathogens. Many of these bacteria exhibit multidrug resistance, rendering a wide range of commonly used antibiotics ineffective [2, 10-12]. Antibiotics that are not fully degraded during use can persist in the environment, including in the food chain, and can be a major source of AMR/ARG transmission. Therefore, monitoring and documenting the rising prevalence of multidrug-resistant bacteria, including those producing extended-spectrum beta-lactamases (ESBL) and AmpC enzymes, in non-hospital environments is crucial to mitigating the public health risks they pose. Understanding how AMR/ARGs spread from non-hospital settings and their impact on human health is essential for enhancing AMR management strategies and preventing the further development and spread of these resistance mechanisms. This study focuses on examining livestock and poultry environments in southeast Nigeria to explore the prevalence of ESBL and AmpC genes in *P. aeruginosa*, *E. coli*, and *Klebsiella* species.

Materials and Methods

Sample collection, culturing, and bacterial identification

This study was conducted with prior approval from the Local Ethics Committee of Enugu State University of Science and Technology (ESUT), Agbani, Nigeria. All procedures adhered to national ethical guidelines for studies involving animal samples. A total of 150 and 300 non-duplicate samples were collected from livestock and poultry environments, respectively, over nine months (January 2021–September 2021) across various farms in southeast Nigeria. The samples were processed using standard microbiological techniques aimed at isolating gram-negative bacteria, including *E. coli, Klebsiella* species, and *P. aeruginosa*, on their specific selective media [13-15].

Detection of ESBL and AmpC production

To identify ESBL and AmpC enzyme production, *E. coli, Klebsiella*, and *P. aeruginosa* isolates were tested using the combined disk diffusion method as previously described [2, 3, 13]. The isolates were cultured on Mueller-Hinton agar and exposed to appropriate antibiotic discs. Third-generation cephalosporins were used to detect ESBL, while second-generation cephalosporins were employed for AmpC detection. The presence of ESBL was confirmed if a ≥ 5 mm increase in the inhibition zone was observed when tested with a combination of ceftazidime or cefotaxime with AMC (20/10 µg) compared to their zones when tested alone. AmpC production was indicated by a characteristic flattening or blunting of inhibition zones for ceftazidime (CAZ), imipenem, or cefotaxime (CTX) discs next to the cefoxitin disc.

PCR detection of resistance genes

Plasmid DNA was extracted from bacterial isolates using the Zymo Plasmid Miniprep Kit (Epigenetics Company, USA). The primers used for PCR amplification of ESBL and AmpC genes are listed in **Table 1**. The DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, USA). The PCR reaction

was carried out in a 50 µl mixture, containing 10 µl of 5x GoTaq, 3 µl of 25 mM MgCl2, 1 µl of 10 mM dNTPs, 1 µl each of forward and reverse primers, 25 µl of DNA Taq (1000 U), and 8 µl of ultrapure water. The PCR conditions included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 96 °C for 30 seconds, 58 °C for 90 seconds, and 72 °C for 60 seconds, with a final extension at 72 °C for 10 minutes.

Table 1. Oligonucleotide primers used for the PCR experiment

Primer name	Primer sequence direction	Amplicon size (bp)
FOX-1 F	AACATGGGGTATCAGGGAGATG	190
FOX-1 R	CAAAGCGCGTAACCGGATTGG	190
blaTEM F	ATGAGTATTCAACATTTCCG	445
*blaTEM*R	CCAATGCTTAATCAGTGAGC	445
blaCTX-M-15 F	CCCATGGTTAAAAAATCACTG	850
blaCTX-M-15 R	CCGTTTCCGCTATTACAAAC	850

Key: F = Forward, R = Reverse

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all ESBL- and AmpC-producing bacterial isolates was assessed in this study. The testing was conducted using a modified version of the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates (Oxoid, UK), adhering to the protocols set by the Clinical Laboratory Standards Institute (CLSI) and previous studies [2, 16-19]. The antibiotics tested included doxycycline (30 µg), colistin sulfate (10 µg), aztreonam (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), sulphamethoxazole-trimethoprim (5 µg), imipenem (10 µg), and mupirocin (300 µg) [Oxoid, UK].

For each bacterial isolate, the inoculum was adjusted to a 0.5 McFarland standard and streaked onto the agar plates, which were then incubated overnight at 37 °C. After incubation, the results were evaluated by comparing the zone diameters to the CLSI standard antibiotic breakpoints, and the bacteria were categorized as either resistant or susceptible based on these criteria [18].

Results and Discussion

This study focused on the prevalence and antibiotic resistance characteristics of Gram-negative bacteria, specifically *E. coli*, *Klebsiella* species, and *P. aeruginosa*, isolated from livestock and poultry environments in southeastern Nigeria. We also investigated their ability to produce resistance mechanisms, such as ESBL and AmpC enzymes, over nine months. Both enteric and non-enteric bacterial species were detected in the samples collected from these environments **(Table 2)**.

Table 2. Isolation of enteric (*E. coli*, *Klebsiella* species) and non-enteric bacteria (*P. aeruginosa*)

Source	***Klebsiella* species** **N (%)**	***P. aeruginosa*** **N (%)**	***E. coli*** **N (%)**
Poultry	8 (3)	5 (2)	42 (14)
Livestock	13 (9)	6 (4)	35 (23)

The most frequently detected bacteria in the samples were *E. coli*, which made up 23% of isolates from livestock and 14% from poultry. *Klebsiella* species and *P. aeruginosa* followed in prevalence **(Table 2)**. **Table 3** illustrates the frequency of ESBL- and AmpC-producing bacteria identified using phenotypic methods in the livestock and poultry samples. For poultry samples, ESBL production was observed in *E. coli* (4%), *Klebsiella* species (1%), and *P. aeruginosa* (2%), while AmpC enzymes were found in *E. coli* (3%), *Klebsiella* species (2%), and *P. aeruginosa* (1%). In livestock samples, ESBL production was detected in *E. coli* (5%), *Klebsiella* species (2%), and *P. aeruginosa* (4%). Meanwhile, AmpC enzyme production was observed in *E. coli* (7%), *Klebsiella* species (3%), and *P. aeruginosa* (6%).

Table 3. Prevalence of ESBL and AmpC-positive bacteria by phenotypic findings

Bacteria	ESBL N (%)	AmpC positive N (%)	ESBL N (%)	AmpC positive N (%)
	Poultry (n = 300)		Livestock (n = 150)	
E. coli	12 (4)	10 (3)	8 (5)	11 (7)
Klebsiella species	2 (1)	5 (2)	3 (2)	4 (3)
P. aeruginosa	5 (2)	3 (1)	6 (4)	9 (6)

Unexpectedly, the occurrence of antimicrobial resistance (AMR) driven by ESBL and AmpC genes in the test bacterial isolates of *E. coli*, *Klebsiella* species, and *P. aeruginosa* rose throughout the 9-month study period **(Figure 1)**.

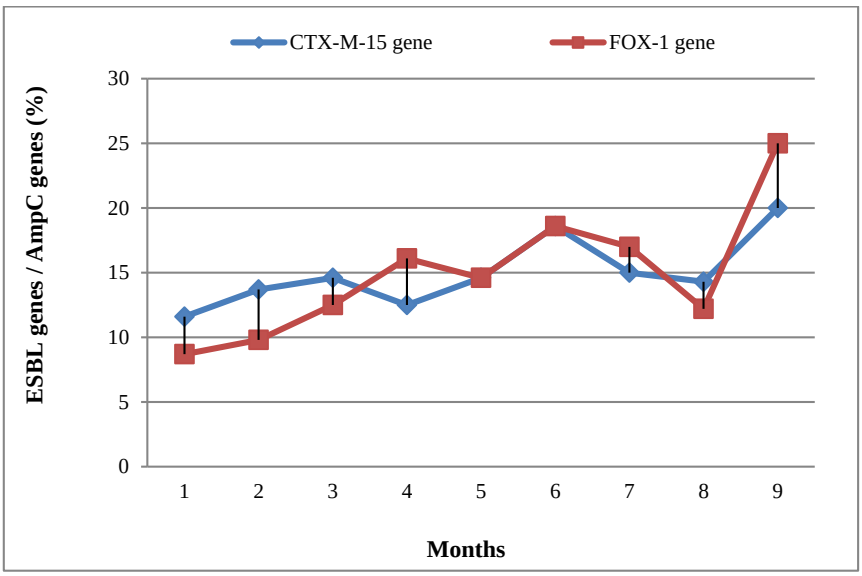

Figure 1. Preliminary results showing the rate of resistance mechanisms in the last year [Jan. 2021–Sept. 2021]. Here we showed, over 9 months in southeast Nigeria, there was a rapid rise in AMR patterns of bacteria caused

Over the nine-month duration of this study conducted in southeast Nigeria, a clear and significant rise in antimicrobial resistance (AMR) associated with ESBL and AmpC genes was observed. Among the different genes identified, the CTX-M-15 gene was the most prevalent ESBL gene found in the bacterial isolates of *E. coli*, *Klebsiella* species, and *P. aeruginosa*. The CTX-M-15 gene is well-known for conferring resistance to cefotaxime, a third-generation cephalosporin that is widely used to treat various bacterial infections in humans. In addition to ESBL genes, the FOX-1 gene emerged as the most commonly detected AmpC gene in these bacterial isolates. The FOX-1 gene is associated with resistance to second-generation cephalosporins, which are critical for treating a range of bacterial infections in clinical settings. These findings highlight the growing challenge of AMR in the region. To further emphasize this, **Figure 2** illustrates the antimicrobial susceptibility profiles of the ESBL- and AmpC-producing bacterial strains against several clinically relevant antibiotics, underscoring the potential impact of this resistance on treatment options.

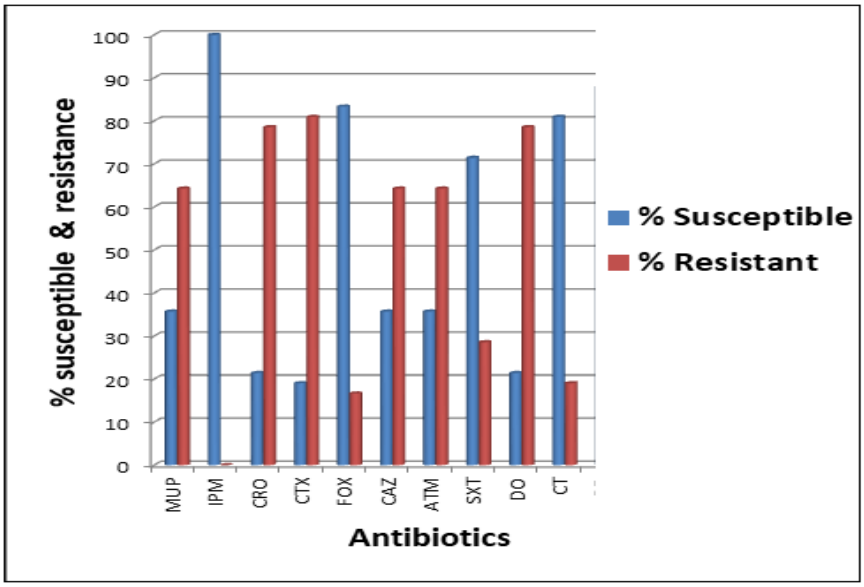

Figure 2. Chart showing antibiogram of the ESBL- and AmpC-producing bacterial isolates; Key: SXT = sulphamethoxazole-trimethoprim, DO = doxycycline, CT = colistin sulphate, ATM = aztreonam, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, FOX = cefoxitin, IPM = imipenem, and MUP = mupirocin

The bacterial isolates producing ESBL and AmpC enzymes from *E. coli*, *Klebsiella* species, and *P. aeruginosa* retrieved from poultry and livestock environments displayed a wide range of susceptibility and resistance patterns to various antibiotics. Notably, a significant portion of these isolates demonstrated resistance to key third-generation cephalosporins, with cefotaxime showing a resistance rate of 81% and ceftriaxone 78%. Other antibiotics, such as mupirocin (64%), doxycycline (78%), ceftazidime (63%), and aztreonam (62%), also exhibited considerable resistance. On the other hand, the most effective antibiotics against these resistant bacterial strains were imipenem (99%), cefoxitin (82%), and colistin sulfate (80%) **(Figure 2)**.

Antimicrobial resistance (AMR) presents a growing threat to global health, with the ability to hinder the control of infectious diseases. Developing countries are particularly vulnerable to the rising challenge of AMR. This issue knows no geographical boundaries and can spread across regions, exacerbating the problem if not adequately addressed. The use of antibiotics in agriculture, especially in animal farming, plays a crucial role in the development and spread of AMR, which in turn contributes to the increase in drug-resistant infections in humans. This study was conducted to explore the prevalence of ESBL- and AmpC-producing *E. coli*, *Klebsiella* species, and *P. aeruginosa* in livestock and poultry farms in southeast Nigeria. The results revealed an increase in the prevalence of these resistant strains, with rates ranging from 3% to 23%, compared to our previous findings on the presence of clinically significant bacteria in non-hospital environments [2, 4].

The bacteria identified in this study are recognized by the World Health Organization (WHO) as priority antibiotic-resistant pathogens, responsible for infections that often require hospital visits, especially in regions with underdeveloped healthcare systems where primary healthcare services are still inadequate. In the poultry environment, phenotypic testing revealed that *E. coli*, *Klebsiella* species, and *P. aeruginosa* produced ESBL and AmpC enzymes at rates of 4%, 1%, and 2% for ESBL, and 3%, 2%, and 1% for AmpC, respectively. These findings suggest that these antibiotic-resistant bacteria may be circulating between animal and human populations, likely due to the unchecked and excessive use of antibiotics in poultry farming.

In contrast, for the livestock environment, the phenotypic detection of ESBL- and AmpC-producing *E. coli*, *Klebsiella* species, and *P. aeruginosa* was observed at 5%, 2%, and 4% for ESBL, and 7%, 3%, and 6% for AmpC-producing isolates, respectively. The prevalence rates of ESBL- and AmpC-producing bacteria from both poultry and livestock were lower than those reported in our earlier study, where the incidence ranged from 10% to 30%. Additionally, these figures were lower than those found in similar studies from Iran [17] and India [16], which reported higher prevalence rates of these resistant pathogens. A study in New Zealand also found comparable prevalence rates of ESBL-producing bacteria in Enterobacteriaceae, including *E. coli* and *Klebsiella* species [20].

Molecular analysis revealed that the CTX-M-15 gene was the most common ESBL gene identified in the bacterial isolates, while the FOX-1 gene was the predominant AmpC gene. CTX-M-15 is a widely disseminated ESBL gene that confers resistance to third-generation cephalosporins, while FOX-1 is an AmpC gene that offers resistance to cephamycin-class antibiotics [6, 12]. These resistance mechanisms render these classes of antibiotics ineffective, contributing to the global challenge of antimicrobial resistance (AMR) [21-25].

The presence of ESBL- and AmpC-producing *Klebsiella* species, *E. coli*, and *P. aeruginosa* in both poultry and livestock environments aligns with prior findings from studies conducted in Italy and the Czech Republic. These studies highlight the spread of CTX-M ESBL and AmpC genes within the broader community, raising concerns about the risk of transmission of these resistant strains from animals to humans [26, 27]. The implications of such transmission are serious, as these resistant bacteria could pose significant challenges in treating human infections. In comparison, other research has shown a broader range of prevalence for ESBL and AmpC gene carriers, ranging from 10 to 40% [28-30]. A separate study in Africa reported a prevalence of ESBL-producing bacteria ranging between 6.7 and 36.1%, which differs slightly from our results [29].

The identification of the CTX-M-15 ESBL gene and FOX-1 AmpC gene in approximately 20% and 25% of *E. coli*, *Klebsiella* species, and *P. aeruginosa* isolates, respectively, is consistent with previous reports. These genes have been identified as the dominant types of ESBL and AmpC enzymes found in both hospital and community settings across Nigeria [2, 3, 31]. A similar high prevalence of CTX-M-15 has been observed in E. coli, Klebsiella species, and P. aeruginosa isolates in countries like China [7]. In Switzerland, a greater prevalence of AmpC genes in E. coli, Klebsiella species, and P. aeruginosa has also been reported [9]. In Korea, CTX-M-15 was one of the most common ESBL types found in E. coli isolates [32], while in Scandinavia, this same ESBL gene was responsible for the spread of multi-resistant Klebsiella pneumoniae strains [33]. The spread of plasmid-mediated ESBL and AmpC genes is especially concerning, as these genes can easily be transferred between bacterial species in both community and healthcare environments.

The susceptibility testing revealed that 78% of the ESBL- and AmpC-positive E. coli, Klebsiella species, and P. aeruginosa isolates were resistant to cefotaxime, ceftriaxone, and doxycycline. Additionally, 64% showed resistance to mupirocin, ceftazidime, and aztreonam. While a few isolates still responded to certain antibiotic classes, particularly third-generation cephalosporins, carbapenems, and some non-beta-lactam drugs, it is well-documented that bacteria expressing ESBL and AmpC enzymes can develop resistance to these antibiotics. This highlights the need to avoid these antibiotics when treating infections caused by such resistant strains. Encouragingly, all the ESBL- and AmpC-producing isolates remained susceptible to imipenem, and about 70% were susceptible to sulphamethoxazole-trimethoprim, cefoxitin, and colistin sulfate.

The findings underscore the importance of ongoing monitoring, surveillance, and reporting of antimicrobial resistance (AMR) in both community and hospital settings. The prevalence of ESBL- and AmpC-producing bacteria in non-hospital environments, particularly in food-producing animals, is an area of growing concern. The spread of these resistant bacteria could pose significant risks to public health, particularly in Nigeria. This study highlights the urgent need for targeted efforts to combat antibiotic resistance in this region, focusing on bacteria that produce ESBL and AmpC enzymes.

Conclusion

The rise of antimicrobial resistance (AMR) has become a significant global health challenge, driven in part by the widespread and often inappropriate use of antibiotics in agriculture, animal farming, and clinical practices, particularly when administered at sub-inhibitory doses. This study explored the prevalence of AmpC and ESBL genes, specifically FOX-1 and CTX-M-15, in *E. coli*, *Klebsiella* species, and *P. aeruginosa* isolated from livestock and poultry environments in southeast Nigeria. Through the use of PCR, which provided accurate detection of these resistance genes, we identified a concerning presence of AmpC and ESBL-producing bacteria in these non-clinical settings. These findings suggest that agricultural and animal farming environments could serve as significant reservoirs for the transmission of AMR genes to human populations. The identification of resistance genes such as CTX-M-15 and FOX-1 underscores the critical need for robust surveillance and monitoring efforts to curb the spread of AMR and ARGs in both the environment and human populations. Without urgent action, the continued evolution of resistant bacteria threatens to undermine the effectiveness of antibiotic therapies, presenting a serious public health risk.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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