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Occurrence of *FOX AmpC* gene among *Pseudomonas aeruginosa* isolates in abattoir samples from south-eastern Nigeria

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In Nigeria, several investigations have been done about the prevalence of the AmpC enzyme in clinical isolates of Gram-negative bacteria; however, little information is available on the occurrence rate of this important enzyme in abattoir specimens that play a major role in the environmental pollution in Nigeria. This study aimed to evaluate the presence of FOX AmpC-producing *Pseudomonas aeruginosa* isolates from abattoir samples by both phenotypic method and polymerase chain reaction (PCR). In this study, 360 abattoir samples were analyzed for the isolation of *P. aeruginosa* strains. Antibiogram was carried out using the disk diffusion technique. The production of AmpC enzymes was phenotypically screened and confirmed using the cefoxitin–cloxacillin double-disk synergy test (CC-DDST). Finally, gene responsible for FOX AmpC enzyme production was investigated using PCR. A total of 147 (40.8%) isolates of *P.* aeruginosa was recovered from the abattoir samples. Ceftazidime and ciprofloxacin with 45.6 and 19% of susceptibility rates were the most and the less effective antibiotics, respectively. A total of 24 (16.3%) *P. aeruginosa* isolates were confirmed to phenotypically produce AmpC enzyme. However, the PCR result showed that only three (12.5%) of *P. aeruginosa* isolates harbored the *FOX AmpC* gene suggesting the attendance of other AmpC resistance genes. This study reported the first occurrence of *P. aeruginosa* isolates harboring the *FOX AmpC* gene in abattoir samples from south-eastern Nigeria. This incident requires the adoption of new policies and measures to prevent the further spread of strains carrying the *AmpC* gene.

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Introduction

As meat products derived from animal abattoir are of particular importance in the human food chain and may serve as reservoirs and propagators of multidrug-resistant (MDR) bacteria, therefore, the study of their microbial contamination is crucial to maintaining public health [1,2]. During the slaughter process, carcass contamination with bacteria occurs by contact with the knives, clothing, equipment, skin, and workers' hands, and the water used for washing carcasses [1]. One of the bacteria reported from meat products around the world, including the African continent, is *Pseudomonas aeruginosa* [3]. *P. aeruginosa* is a notable Gram-negative bacterial pathogen responsible for most nosocomial and community-acquired infections around the world [4].

Also, *P. aeruginosa* strains encode genes that mediate varieties of β-lactamase enzymes, such as AmpC enzymes that hydrolyze and confer resistance to β-lactam antibiotics [5,6]. AmpC enzymes are active against cephamycins as well as oxyimino-β-lactams; this differentiates them from extended spectrum β-lactamases (ESBLs) that notably confer the resistance to the third-generation cephalosporins [7]. In addition to their chromosomal locations, β-lactamase AmpC-encoded genes have been identified on the plasmids in *P. aeruginosa* isolates, therefore, horizontal transmission of plasmids could help to prompt spread of this enzyme among diverse Gram-negative bacteria [6]. *P. aeruginosa* isolates that harbor the AmpC enzyme are resistant to a wide range of antimicrobial drugs, so, they considered as a serious problem in the medical therapeutics field [6]. Despite the numerous studies on the epidemiology of AmpC enzymes in clinical isolates of *P. aeruginosa* across the world, however, little studies have focused on nonclinical isolates. As nonclinical specimens, such as abattoir samples play an important role in the spreading of antibiotic resistance (ABR), it is necessary to investigate the resistance genes in these environments to take preventive measures [8]. In Nigeria, most of the investigations regarding prevalence of AmpC enzyme are restricted to clinical isolates of Gram-negative bacteria, mainly *Enterobacteriaceae* family, and little information is available on this important enzyme in nonclinical specimens, especially abattoir specimens that play a major role in environmental pollution in Nigeria leading to serious health problems [9,10]. According to the above, Nigeria must develop and domesticate strategies of detecting, reporting, and monitoring the emergence and spread of AmpC-producing bacteria in this part of the world. This study aimed to characterize the occurrence of *AmpC* gene in *P. aeruginosa* isolates that were collected from abattoir samples in Ebonyi state, south-eastern Nigeria using phenotypic method and PCR.

Materials and methods

Ethical clearance

This study was approved by the local ethics committee of Ebonyi State University, Abakaliki, Nigeria. Samples and isolates were processed and handled according to all relevant national and international guidelines.

Isolation and characterization of *Pseudomonas aeruginosa*

A total of 360 cow abattoir samples were collected in Abakaliki metropolis, Ebonyi State, south-eastern Nigeria during January to May 2016. All samples were cultured in 5 ml of nutrient broth (Oxoid, Hampshire, UK) and incubated overnight at 37 °C. A loopful of the specimen or turbid solution was plated aseptically onto cetrimide agar (Oxoid) plates for the selective isolation of *P. aeruginosa*. The culture plates were incubated at 37 °C for 18–24 h. The presence of *P. aeruginosa* was determined qualitatively based on the production of pyocyanin and pyoverdin pigments on cetrimide selective media [11]. Single colony of the suspected *P. aeruginosa* isolates from the cetrimide agar plates was purified on freshly prepared nutrient agar (Oxoid), and the positive culture was selected for biochemical testing using oxidase test, methyl red test, Voges–Proskauer test, citrate utilization, oxidative fermentative test, and growth at 42 °C [12]. *P. aeruginosa* ATCC 10145 was used as quality control strain.

Antibiotic susceptibility testing

The Kirby–Bauer disk diffusion method was used for antibiotic susceptibility studies on Mueller–Hinton agar plates (Oxoid) as described by the Clinical and Laboratory Standards Institute (CLSI) [13]. Antibiotic disks (MAST Diagnostics, Merseyside, UK) constituting imipenem (10 μg), meropenem (10 μg), ertapenem (10 μg), cefoxitin (30 μg), ceftazidime (30 μg), amikacin (10 μg), gentamicin (10 μg), cefotaxime (30 μg), ceftriaxone (10 μg), ciprofloxacin (10 μg), ofloxacin (10 μg), and aztreonam (30 μg) were each aseptically placed at a distance of 15 mm apart on Mueller–Hinton agar plates already inoculated with the *P. aeruginosa* isolates (adjusted to 0.5 McFarland turbidity standards). The Mueller–Hinton agar plates were incubated at 37 °C for 18–24 h. Inhibition zone diameters were recorded and interpreted using the antibiotic breakpoints of the CLSI 2016 as a guideline [13]. *P. aeruginosa* ATCC 27853 was used as a quality control strain.

Screening and phenotypic confirmation of AmpC β-lactamase production

P. aeruginosa isolates were phenotypically screened for AmpC enzyme production using cefoxitin (30 μg) disk. Isolates showing reduced susceptibility to cefoxitin disk (30 μg) were considered as potential AmpC enzyme producers [14,15]. Cefoxitin–cloxacillin (30/200 μg) (Liofilchem, Roseto degli Abruzzi, Italy) double-disk synergy test (CC-DDST) was used as the confirmatory

test for AmpC enzyme detection in the potential AmpC-producing *P. aeruginosa* strains [14]. All tests were performed on Mueller–Hinton agar plates already inoculated with the test *P. aeruginosa* isolate (equivalent to 0.5 McFarland turbidity standards). The plates were incubated at 37 °C for 18–24 h, and then the inhibition zones were recorded. A difference of at least 4 mm in the cefoxitin–cloxacillin inhibition zones compared with the cefoxitin disk used alone was phenotypically confirmed the AmpC enzyme production [14].

DNA preparation

The extraction of bacterial total DNA from *P. aeruginosa* isolates was carried out using the GENESpin DNA isolation kit (Eurofins, Hamburg, Germany) according to the manufacturer's guidelines. The extracted DNA samples were later stored at −20 °C for the PCR evaluation.

PCR detection of *AmpC FOX* gene

The PCR amplification of FOX *AmpC β-lactamase* gene was carried out using the specific primers synthesized and supplied by Inqaba Biotechnical Industries Ltd (Inqaba Biotechnical Industries Ltd, South Africa) in a thermal cycler (Lumex instruments, Canada) according to previously described protocols [9]. A final PCR mixture of 26.5 µl containing 0.2 µl of Taq polymerase enzyme U/µl, 2.5 µl of 10X PCR buffer along with 2.5 µl $MgCl_2$, 1 µl of 10 pmol/l from each of the forward and reverse primers, 2.5 µl of dNTPs (2 mmol/l), 3 µl of DNA template (from the test isolates), 14.8 µl of nuclease-free water were used for PCR amplification. The specific primers for *FOX* gene amplification were: FOXM-F (AACATGGGGTATCAGGGAGATG) and FOXM-R (CAAAGCGCGTAACCGGATTGG). Amplification conditions include initial denaturation at 94 °C for 3 min, followed by 25 cycles of DNA denaturation at 94 °C (30 s), annealing at 64 °C (30 s), extension at 72 °C (1 min), with a final extension at 72 °C for 5 min. Gel electrophoresis of the 190 bp PCR products was carried out using 1.5% agarose gel (Inqaba Biotechnical Industries Ltd) for 1 h at 80 V. The visual output of the amplicons was captured using an electrophoresis photography system (Fotodyne Foto/Analyst Investigator, Fotodyne, Japan). A FOX AmpC carrying *E. coli* from the previous study was used as a positive control [16].

Statistical methods

Data analysis was carried out with the Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS, Chicago, Illinois, USA). Data were expressed as numbers and percentages.

Results

Characterization of *Pseudomonas aeruginosa*

Out of the 360 abattoir samples analyzed in this study, a total of 147 (40.8%) isolates of *P. aeruginosa* were recovered.

Table 1. Antibiotic susceptibility testing of *Pseudomonas aeruginosa* isolates.

Antibiotics (µg)	Susceptible [*N* (%)]	Resistant [*N* (%)]
CRO (30)	52 (35.4)	95 (64.6)
FOX (30)	29 (19.7)	118 (80.3)
IPM (10)	49 (33.3)	98 (66.7)
CAZ (30)	67 (45.6)	80 (54.4)
ETP (30)	57 (38.8)	90 (61.2)
OFX (10)	54 (36.7)	93 (63.3)
CN (10)	65 (44.2)	82 (55.8)
AK (10)	53 (36.1)	94 (63.9)
CIP (10)	28 (19.0)	119 (81.0)
CTX (30)	30 (20.4)	117 (79.6)
MEM (10)	58 (39.5)	89 (60.5)
ATM (30)	61 (41.5)	86 (58.5)

AK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; CRO, ceftriaxone; CTX, cefotaxime; ETP, ertapenem; FOX, cefoxitin; IPM, imipenem; MEM, meropenem; OFX, ofloxacin.

Antibiotic susceptibility testing

The *P. aeruginosa* isolates showed varying rates of resistance to the tested antibiotics used in this study (Table 1). The antibiotic resistance pattern evaluation showed a high resistance rate more than 50% against carbapenems including imipenem (66.7%), ertapenem (61.2%), and meropenem (60.5%). Reduced susceptibility of the *P. aeruginosa* isolates was also observed against ciprofloxacin (81.0%), cefoxitin (80.3%), and cefotaxime (79.6%). Ceftazidime and ciprofloxacin with 45.6 and 19.0% of susceptibility rates were the most and the less effective antibiotics, respectively. The MDR pattern (resistant to $\geq$1 agent in $\geq$3 antimicrobial categories) was seen in 88.4% (130/147) of isolates.

Phenotypic screening of AmpC β-lactamase

The result of the phenotypic detection of AmpC enzyme showed that 118/147 (80.3%) of *P. aeruginosa* isolates, were resistant against cefoxitin from which 24 isolates were confirmed as an AmpC producer by the CC-DDST method.

PCR assay for *FOX AmpC* gene

Overall, *FOX AmpC* gene was detected in a total of 3/24 (12.5%) of phenotypically AmpC-positive *P. aeruginosa* isolates by PCR technique. The *P. aeruginosa* isolates harboring the *FOX AmpC* gene were also found to be multidrug-resistant to antibiotics in the following classes: fluoroquinolones, cephalosporin, carbapenems, aminoglycosides, and cephamycin.

Discussion

High AmpC-producing Gram-negative bacteria are generally resistant to multiple antibiotics [17]. This resistance profile severely restricts the effective treatment options available against these microorganisms. Nowadays, the issue of multidrug-resistant bacteria is not only

important from the clinical point of view but also the spread of these dangerous microorganisms in various environments, including food industries, has created a new challenge for health communities [18]. Therefore, a periodic surveillance program is essential for any country to prevent the more spread of these bacteria.

In this study, a total of 147 (40.8%) isolates of *P. aeruginosa* collected from abattoir samples in Ebonyi state, south-eastern Nigeria were investigated for their antibiotic resistance patterns and occurrence of AmpC production. In this study, the frequency of *P. aeruginosa* was determined by 40.8% (147/360), which was higher compared with the previous report by Elhariri *et al.* [19], who indicated 22.5% (45/200) of prevalence in two major abattoirs from Egypt. This high prevalence of *P. aeruginosa* makes the necessity of assuming new policies for prevention and control of further spreading of this pathogen in the food industry in Nigeria.

In the present study, the results of AST revealed a high resistance rate of more than 50% against all antibiotic categories. This high percentage of ABR in abattoir samples from Nigeria was a warning sign to make new control plans to prevent the spread of multidrug-resistant bacteria. The result of this study was in line with the previous report from Africa that suggested the ABR as a serious public health concern in the food chain of the Nigerian population [2]. In this study, the resistance rate against ciprofloxacin, meropenem, amikacin, and imipenem was higher than the previous studies reported from Egypt and south-western Nigeria [19,20]. More so the high resistance level to the third-generation, such as cefotaxime and ceftriaxone was worrisome. However, contrary to previous studies from Nigeria and other African countries where carbapenem categories were reported to be the most effective drugs against *P. aeruginosa* [19,20], the ceftazidime was the most effective in this study. This high resistance rate to carbapenems family (more than 55%) in this study paves the way for investigating the mechanisms contributed to this resistance and highlights the necessity for future screening of involved carbapenemases in abattoirs and food-producing animals of our region. Currently, infections caused by MDR pathogens are on the rise worldwide, limiting treatment options. In the current study, 88.4% of *P. aeruginosa* isolates were MDR that was a predictable result based on previous studies from Nigeria and elsewhere in Africa [19,21].

In this study, we performed the CC-DDST method and PCR to evaluate the occurrence of AmpC in *P. aeruginosa* isolates. To the best of our knowledge, this study was the first effort to the molecular determination of FOX AmpC-producing *P. aeruginosa* isolates in abattoir samples in south-eastern Nigeria. There is generally no comprehensive information on the prevalence of this enzyme in *Pseudomonas* strains in abattoir environments, especially in the African continent. However, most reports of this enzyme are from other Gram-negative bacteria, especially *Escherichia coli* [16,19]. Overall, most of the existing investigations are from Europe indicate the occurrence of AmpC-producing *E. coli* ranging between 0 and 13%. Data from Asia and America are limited (range for AmpC 0–95%), whereas those from Africa and Australia are lacking [22]. The production of AmpC was detected in 16.3% (24/147) of isolates using CC-DDST method in this study. This result was lower than previous studies performed in Nigeria and elsewhere, particularly compared with the *Enterobacteriaceae* family [19,23]. Also, *FOX AmpC* gene was identified in 3 isolates of 24 phenotypically AmpC-positive *P. aeruginosa*. This difference between the results of the PCR and CC-DDST method may be aroused from the false-negative and false-positive phenomenon related to the phenotypic method [9]. The CLSI guidelines do not yet have an approved screening and validation test for AmpC detection. In light of the studies, although some phenotypic tests can be used for this purpose, phenotypic tests do not have standardization to detect the presence of AmpC alone [24]. The method of detection of AmpC, which is considered the gold standard by some researchers, is currently the PCR technique [25]. However, PCR is not practical and inexpensive enough considering accessibility and laboratory facilities. Therefore, there is a need for standardized phenotypic methods that can be applied under routine laboratory conditions. Other possibilities for the difference between the results of PCR and CC-DDST may be contributed to the presence of other potential AmpC types that were not investigated in this study and can be considered as one of the limitations of current investigation. Six main families of AmpC β-lactamase, including MOX, CIT, DHA, EBC, FOX, and ACC, have so far been identified in Gram-negative bacteria [26]. In previous reports from North America and Asia, the CMY-2 AmpC had a very high prevalence [22]. Another limitation of this study was the lack of sequencing of the *AmpC* gene, because of the lack of funding.

Conclusion

The present study affords an outlook into the antibiotic susceptibility patterns and AmpC production of *P. aeruginosa* isolates from abattoir samples in south-eastern Nigeria. The MDR phenotype and detection of AmpC enzyme among the *P. aeruginosa* isolates in this study established their potential risk for human health. Thus, urgent measures are necessary to restrict the spread of antibiotic resistance bacteria especially in livestock. Further studies will be carried out in continuation, to fill the pending gaps in this study, such as screening of other AmpC types and extended-spectrum-β-lactamases among the *P. aeruginosa* from the animals sources.

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Conflicts of interest

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