

Phenotypic Detection of AmpC Beta-Lactamase among Anal *Pseudomonas aeruginosa* Isolates in a Nigerian Abattoir

Ejikeugwu Chika¹,
Esimone Charles²,
Iroha Ifeanyichukwu¹,
Ugwu Chigozie²,
Ezeador Chika³,
Duru Carissa² and
Adikwu Michael⁴

Abstract

Pseudomonas aeruginosa is an important human pathogen which causes a variety of infections that are often difficult to treat due to its resistance to many antibiotics including the beta-lactams. The organism is notorious for being intrinsically resistant to many antimicrobial agents by exhibiting low permeability of its outer membrane, the constitutive expression of various efflux pumps and the naturally occurring chromosomal AmpC β lactamase. The organism can also acquire additional resistant genes from other resistant pathogens via genetic transfer mechanisms. This presumptive study evaluated the occurrence of *P. aeruginosa*-producing AmpC beta-lactamase enzymes from an abattoir in Abakaliki metropolis, Ebonyi State, Nigeria. A total of 75 fecal swab samples from the anal region of cows were bacteriologically analyzed for the isolation of *P. aeruginosa* isolates using ceftrimide selective agar (supplemented with glycerol). AmpC enzyme production was phenotypically detected by the disk approximation/three-dimensional method using ceftazidime disk (30 μ g), ceftazidime (30 μ g) and cefotaxime disk (30 μ g). A total of 25 *P. aeruginosa* isolates were bacteriologically isolated from the anal swab samples. And the *P. aeruginosa* isolates were highly resistant to ceftriaxone, ceftazidime, ceftazidime, ceftazidime, gentamicin, ampicillin, sulphamethoxazole-trimethoprim and cefepime. Ertapenem, nitrofurantoin and aztreonam exhibited antimicrobial activity against the *P. aeruginosa* isolates. A total of 9 (36 %) *P. aeruginosa* isolates were phenotypically confirmed to produce AmpC beta-lactamase by the disk approximation method. This study further gives impetus to the potential reservoir of antibiotic resistant bacteria in livestock. *P. aeruginosa* producing AmpC beta-lactamases in the community portends danger for the healthcare sector because such pathogens could spread to the hospital environment via community-acquired infections when patients report to the hospital for medical attention and become hospitalized. It is therefore vital to control and restrict the usage of antibiotics in the community especially in the rearing of livestock and breeding of poultry birds in order to abate the emergence and spread of drug resistant bacteria in the community.

Keywords: *Pseudomonas aeruginosa*; AmpC enzymes; Multidrug resistance; Abattoir Nigeria

- 1 Department of Applied Microbiology, Faculty of Sciences, Ebonyi State University, Abakaliki, Ebonyi State, Nigeria
- 2 Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria
- 3 Department of Medical Microbiology and Parasitology, Faculty of Medicine, Nnamdi Azikiwe University Teaching Hospital, Nnewi Anambra State, Nigeria
- 4 University of Abuja Main Campus, Abuja, Airport Road, Abuja, Nigeria

Corresponding Author: Ejikeugwu Chika

Department of Applied Microbiology,
Faculty of Sciences, Ebonyi State University,
Abakaliki, Ebonyi State, Nigeria.

✉ ejikeugwu_chika@yahoo.com

Tel: +2348097684562

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Introduction

The menace posed by multidrug resistant bacteria including *Pseudomonas aeruginosa* positive for AmpC enzyme production necessitates the need to detect by molecular and phenotypic characterization the presence of AmpC β -lactamases from both environmental and clinical isolates-owing to the fact that

antibiotic resistance is an increasing problem in our health sector worldwide. AmpC β -lactamases are clinically important cephalosporinases encoded on the chromosomes of members of the *Enterobacteriaceae* family and a few other organisms including *Pseudomonas aeruginosa*, where they mediate resistance to cephalothin, ceftazidime, ceftazidime, most penicillins, and β -lactamase inhibitor- β -lactam combinations [1]. AmpC

enzymes are inducible and can be expressed at high levels by mutation in other bacteria and the overexpression of AmpC enzymes in bacteria can confer resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone and this is a problem especially in infections due to *Enterobacter aerogenes* and *Enterobacter cloacae*, where an isolate initially susceptible to these agents may become resistant upon therapy [1]. AmpC β -lactamase of *Escherichia coli* was the first bacterial enzyme reported to destroy penicillin [1]. Organisms producing these multidrug resistant enzymes could easily be disseminated in the community via livestock products especially through consumption of meat and other infected animal products. Direct body contact with infected animals could also serve as route via which AmpC-producing bacteria could be disseminated in the community; and this could lead to the emergence of community-acquired infection that is difficult to treat. AmpC β -lactamase production is one of the mechanisms of resistance to β -lactam antibiotics in Gram negative bacteria conferring resistance to a wide variety of β -lactam antibiotics. *P. aeruginosa* is a nosocomial pathogen that is implicated in many clinical conditions and *P. aeruginosa* isolates that produces AmpC enzymes portend great clinical implications owing to the mortality and morbidity caused by multidrug resistant *P. aeruginosa* isolates [2-4]. AmpC enzymes are active on cephamycins as well as oxyimino- β -lactams and this differentiates them from other antibiotic hydrolyzing enzymes including extended spectrum beta-lactamases (ESBLs) [5]. It is usually encoded by bacterial chromosomal genes in many Gram negative bacilli, though some are plasmid mediated as aforementioned. The AmpC enzyme in *E. coli* is poorly expressed and the AmpC gene is missing from the chromosome of *Klebsiella* and *Salmonella* species [5]. Characteristically, AmpC β -lactamases are known to mask ESBL production in organisms harbouring both AmpC and ESBLs, and they are poorly inhibited by clavulanic acid. AmpC enzymes are also inhibited by fourth generation cephalosporins (such as cefepime) and the carbapenems (e.g. imipenem and meropenem) and their production can either be caused by mutation or as a result of an inducing agent. Bacterial organisms producing AmpC β -lactamase and other multidrug resistant genes such as ESBLs are resistant to virtually all β -lactam antibiotics and some non- β -lactam antibiotics as well [6,7]. Several studies abound that show the frequency of AmpC beta-lactamases in Gram negative bacteria from livestock such as cow and swine and even from dogs-which are generally known as companion animals [8-10]. This call for the need to screen community isolates or samples for the presence of AmpC positive bacteria since these organisms also exist in the community. The plan will help to abate the spread of community-acquired plasmid-encoded AmpC mediated resistance in the hospital environment and even in the community. Multidrug resistant organisms continue to be a major problem for health personnel's around the world as their menace are gradually eroding the efficacy of our therapeutic armamentarium. It is therefore very important for microbiology laboratories all over the world to detect these organisms in their routine susceptibility studies using prompt and accurate detection techniques. It is in view of this that this study was aimed at presumptively determining by phenotypic method

the occurrence of *P. aeruginosa* isolates that produces AmpC beta-lactamases in the community.

Materials and methods

Sample Collection

Seventy five (75) fecal swab samples from the anal region of cows were collected from an abattoir prior to slaughter. The samples were collected using sterile swab sticks and transferred into their containers after collection. All samples were properly labeled and transported immediately to the Microbiology Laboratory of Ebonyi State University, Abakaliki, Nigeria for bacteriological analysis.

Isolation and Characterization of Organism

Cetrimide selective agar (constituted with glycerol) was used for the selective isolation of *P. aeruginosa* from the test samples. Each of the anal swab samples was inoculated in 5 ml tryptone soya broth for 18-24 hours at 37°C. A loopful of the overnight broth culture was streaked on cetrimide selective agar and the plates were incubated at 37°C for 18-24 hours. Suspect colonies were inoculated onto freshly prepared cetrimide selective agar for the isolation of pure cultures of *P. aeruginosa*. *P. aeruginosa* produces greenish colouration on cetrimide selective agar. The suspect *P. aeruginosa* were further characterized using standard microbiological identification techniques as was described previously [11].

Antibiogram

All the *Pseudomonas aeruginosa* isolates were tested for susceptibility to some commonly used antibiotics using the Kirby-Bauer disk diffusion method. The antibiotics used include: Gentamicin (CN), Cefotaxime (CTX), Sulphamethoxazole-Trimethoprim (SXT), Ceftriaxone (CRO), Cefepime (FEP), Ampicillin (AMP), Cefoxitin (FOX), Ertapenem (ETP), Nitrofurantoin (F) and Aztreonam (ATM). Each of the antibiotics disk were aseptically placed on Mueller-Hinton (MH) agar plates previously inoculated with the test organism and the plates were incubated at 37°C for 18-24 hrs. After incubation, the Inhibition Zone Diameter (IZD) was measured using a meter rule and recorded as per the guidelines of the Clinical Laboratory Standard Institute (CLSI) [12,13].

Screening for AmpC beta-lactamase production

The isolated *Pseudomonas aeruginosa* isolates were screened for AmpC enzyme production by testing for their susceptibility to cefoxitin. Isolates that showed reduced susceptibility to the antimicrobial activity of cefoxitin were suspected to produce AmpC enzyme when their zones of inhibition were ≤ 18 mm; and this warranted phenotypic confirmation [14].

Evaluation of AmpC β -lactamase Production

AmpC β -lactamase production was phenotypically detected on only isolates that showed reduced susceptibility to cefoxitin according to a previously described methodology [14,15]. To test for AmpC enzyme production, ceftazidime (30 μ g) and cefotaxime (30 μ g) disks were each placed at a distance of 20 mm from

cefoxitin (30 µg) disk on a MH agar plate already inoculated with the test *P. aeruginosa* isolate. The production of AmpC enzyme by the test isolate was phenotypically confirmed when the zones of inhibition produced by either of the cephalosporins (ceftazidime or cefotaxime) when used in conjunction with cefoxitin were ≥ 5 mm. AmpC positive isolates also show blunting of ceftazidime or cefotaxime zone of inhibition adjacent to the cefoxitin disc [14,15].

Quality control organism

Quality control as per the antimicrobial susceptibility studies was done with *P. aeruginosa* ATCC 10145 (Oxoid, UK).

Results

Out of the seventy five (75) fecal swab samples from the anal region of cows that was analyzed in this present day study, 25 *P. aeruginosa* isolates was isolated. *P. aeruginosa* is commonly isolated from wound and burn infections, and also from moist regions in the hospital and non-hospital environments. And it is also isolated from abattoirs as well. The frequency of isolation of *P. aeruginosa* isolates from the fecal swab samples obtained from the anal region of cow is shown in **Table 1**. Antimicrobial susceptibility studies was also performed on all the isolated *P. aeruginosa* isolates recovered in this study using Gentamicin (CN), Cefotaxime (CTX), Sulphamethoxazole-trimethoprim (SXT), Ceftriaxone (CRO), Cefepime (FEP), Ampicillin (AMP), Cefoxitin (FOX), Ertapenem (ETP), Nitrofurantoin (F) and Aztreonam (ATM). The antibiotic susceptibility test result (antibiogram) of the *P. aeruginosa* isolates to some commonly used antibiotics is shown in **Table 2**. The *Pseudomonas aeruginosa* isolates were highly resistant to the third generation cephalosporins, ceftazidime (92 %) and ceftriaxone (84 %). *P. aeruginosa* isolates were also resistant to gentamicin (92 %), ampicillin (88 %), and cefoxitin (80 %). Reduced susceptibility of the test organism was also recorded against sulphamethoxazole-trimethoprim (32 %), and ertapenem

Table 1 *P. aeruginosa* recovery on cetrimide selective agar.

Samples	No. of samples	No. of <i>P. aeruginosa</i> isolates (%)
Feecal swab samples from anal swab of cow	75	25 (33.3)

Table 2 Antibiogram of the isolated *P. aeruginosa* isolates

Antibiotics (µg)	No. (%) of susceptible isolates	No. (%) of resistant isolates
Ceftazidime (30)	2 (8)	23 (92)
Gentamicin (10)	2 (8)	23 (92)
SXT (25)	17 (68)	8 (32)
Ceftriaxone (30)	4 (16)	21 (84)
Cefepime (FEP)	13 (52)	12 (48)
Ampicillin (AMP)	3 (12)	22 (88)
Cefoxitin (30)	5 (20)	20 (80)
Ertapenem (10)	15 (60)	10 (40)
Nitrofurantoin (10)	23 (92)	2 (8)
Aztreonam (30)	18 (72)	7 (28)

SXT= sulphamethoxazole-trimethoprim

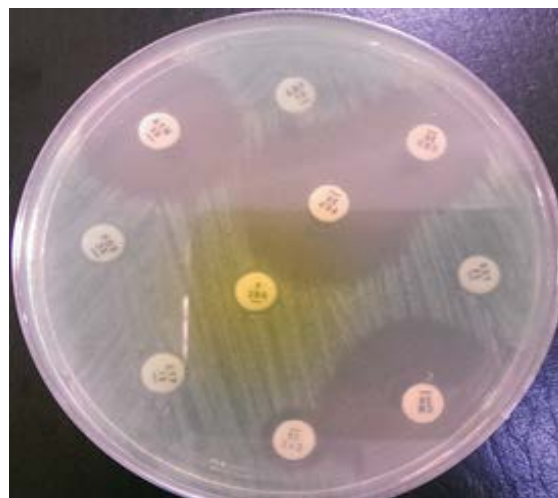


Figure 1 Illustration of susceptibility test plate of a *P. aeruginosa* isolate. The test antibiotics are gentamicin (CN), Cefotaxime (CTX), Sulphamethoxazole-trimethoprim (SXT), Ceftriaxone (CRO), cefepime (FEP), Ampicillin (AMP), Cefoxitin (FOX), Ertapenem (ETP), Nitrofurantoin (F) and Aztreonam (ATM). The *P. aeruginosa* isolate is susceptible to gentamicin, ceftriaxone, cefepime, and aztreonam, but resistant to cefoxitin, ertapenem, ampicillin, cefotaxime and sulphamethoxazole-trimethoprim.

(40 %). However, aztreonam, cefepime and nitrofurantoin were effective against the *P. aeruginosa* isolates at percentage levels of 72 %, 52 % and 92 % respectively (**Figure 1**).

Out of the 25 isolates of *P. aeruginosa*, only 9 isolates were phenotypically confirmed to produce AmpC beta-lactamases. However, sixteen (16) isolates of *P. aeruginosa* were confirmed to be AmpC beta-lactamase non-producers (**Table 3**).

Discussion

P. aeruginosa is one of the major bacterial pathogens responsible for a handful of nosocomial infections including but not limited to cystic fibrosis, otitis media and wound infections; and the organism has several resistant mechanisms that allows it to thrive even in the face of potent antimicrobial onslaught. The organism is widespread in nature, and it can be regularly found in soils, surface waters, moist environments and in small numbers in the intestines of animals [11,13]. The detection of AmpC production in bacterial pathogens is important for ensuring effective antibiotic therapy since the presence of an AmpC beta-lactamase frequently seems to result in therapeutic failure when broad-spectrum cephalosporins are used [5,6]. AmpC beta-lactamase production in Gram negative bacteria including *P. aeruginosa* isolates from both the community and hospital environment is fast becoming a global threat and the emergence and spread of these multidrug resistant organisms is a threat to available drugs. Antibiotic resistance occurs when antibiotics are over prescribed, misused, and even when people fail to practice good personal hygiene. Of most importance is the fact that antibiotics are now used for the rearing of livestock and even in fish farming;

Table 3 Distribution of AmpC beta-lactamase among the *P. aeruginosa* isolates

Organism	AmpC producers n(%)	AmpC non-producers n(%)
<i>P. aeruginosa</i>	9 (36)	16 (64)

and this practice (though beneficial to the farmers and their animals) allows microbes to mutate and become resistant to these drugs. This study phenotypically evaluated the occurrence of AmpC beta-lactamase-producing *P. aeruginosa* isolates from fecal swabs that originated from the anal region of cows in a local abattoir in Abakaliki metropolis, Nigeria. Out of the 75 fecal swab samples analyzed in this study, only twenty five isolates of *P. aeruginosa* were recovered from the samples and these organisms were screened and confirmed phenotypically for the production of AmpC beta-lactamases. The antimicrobial susceptibility test result showed that nitrofurantoin, ertapenem, sulphamethoxazole-trimethoprim and aztreonam were the most active antibiotics against the *Pseudomonas aeruginosa* isolates tested. Cefepime, a fourth generation cephalosporin was also active against the *P. aeruginosa* isolates. Previous studies have shown that cefepime has activity against bacterial organisms that produce AmpC beta-lactamase [1-4]. It was also found in our study that the *P. aeruginosa* isolates were highly resistant to ceftriaxone, ceftazidime, ampicillin, gentamicin and ceftazidime. *P. aeruginosa* isolates are naturally resistant to antimicrobial agents due to the composition of their cell walls. Several other studies have also reported the low susceptibility of *P. aeruginosa* to some commonly used antibiotics [2,13-15]. The low levels of *P. aeruginosa* isolates to antibiotics (as reported in this study) is in accordance to a similar work conducted in Egypt – where *P. aeruginosa* isolates were also found to be resistant to ampicillin, gentamicin, ceftazidime, aztreonam, and cefepime [15,16].

A total of 9 (36 %) *P. aeruginosa* isolates were phenotypically confirmed to produce AmpC beta-lactamase in our presumptive study. In 2012, Akinniyi *et al.* [16] reported the production of AmpC enzymes in enteric bacteria in Abeokuta, Nigeria. Previous studies on plasmid-mediated and/or chromosomal AmpC beta-lactamase production in bacteria have mainly been conducted on clinical isolates [9-16]. Only few studies in this region elucidated the production of AmpC enzymes in *P. aeruginosa* isolates originating from community samples. However, AmpC beta-lactamase production in both enteric and non-enteric bacteria is of clinical significance because of their resistance to some available antibiotics used in clinical medicine. Conclusively, this present day study have presumptively shown that *P. aeruginosa* isolates from the community exhibit a high level of resistance to different classes of antibiotics and that these organisms produce AmpC beta-lactamase enzymes – which gives the organism the ability to be resistant to a wide variety of antibiotics including beta-lactams and some non-beta-lactam antibiotics. *P. aeruginosa* produces a wide variety of beta-lactamases. However, this present study only focused on AmpC enzyme production by *P. aeruginosa*. The continuous monitoring of the antibiotic usage in the community (especially in agricultural practices such as in livestock rearing) via proper antimicrobial susceptibility studies on community isolates including *P. aeruginosa* isolates is paramount to containing the emergence and spread of drug resistant bacteria in the community.

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