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Research Article
Antibiogram and Molecular Characterization of AmpC and ESBL-Producing Gram-Negative Bacteria from Poultry and Abattoir Samples

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Abstract

Background and Objective: The global antibiotic resistance threat posed by ESBL and AmpC-producing Gram-Negative Bacteria (GNB) is a public health menace that rolls back the gains of 'One Health'. This study investigated the antibiogram and prevalence of AmpC and ESBL genes in *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas* spp. from poultry and abattoir milieus in Enugu and Ebonyi States, Nigeria. **Materials and Methods:** Isolation, identification and characterization of GNB from samples (150 abattoirs and 300 poultry) were done using standard microbiological techniques. Antimicrobial Susceptibility Testing (AST), as well as phenotypic screening for ESBL and AmpC enzymes, was performed using the Kirby-Bauer disc diffusion technique. PCR technique was used to screen isolated GNB for AmpC and ESBL genes. **Results:** Exactly 42 *E. coli* and 8 *Klebsiella* spp. isolate from poultry samples and another 5 *P. aeruginosa* isolates from abattoir samples were phenotypically confirmed to be ESBL-producers. AmpC enzymes were phenotypically detected in 8 *E. coli* and 13 *P. aeruginosa* isolates from poultry samples. All ESBL and AmpC-positive bacteria exhibited high resistance frequencies to tested antibiotics, especially to the carbapenems and cephalosporins. ESBL genes (CTX-M, SHV-1, TEM) and AmpC genes (ACC-M, MOX-M, DHA-M) were harbored by the isolated GNB in this study. Overall, the DHA-M and CTX-M genes, mediating AmpC and ESBL production respectively were the most prevalent genes harbored by the tested GNB. **Conclusion:** This study reported that AmpC and ESBL genes are harbored by Gram-negative bacteria (*E. coli*, *Klebsiella* species and *P. aeruginosa*) that emanated from poultry and abattoir milieus.

Key words: Gram-negative bacteria, antibiotic-resistant bacteria, abattoir, poultry, resistance genes, AmpC, ESBL

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Globally, antibiotic-resistant bacteria still pose a threat to public health. The increasing prevalence of antibiotic-resistant microorganisms, especially those with multidrug resistance mechanisms (such as extended-spectrum beta-lactamases, AmpC and Metallo-beta-lactamases) is of global concern as they are known to make the treatment of bacterial related infections difficult[1]. The presence of beta-lactamase enzymes in a clinical setting plays a very critical role in the selection of appropriate therapy because the majority of the antibiotics (e.g., cephalosporins, carbapenems and penicillins) they hydrolyze are included in the preferred treatment regimens for treat many bacterial related infections[2]. Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated β-lactamase enzymes that hydrolyze and confer resistance to broad-spectrum cephalosporins but are inhibited by clavulanic acid[1,3]. On the other hand, AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many Enterobacteriaceae and a few other bacteria where they mediate resistance to some cephalosporins and most penicillins[1]. The presence of resistance genes that mediate the production of ESBLs and AmpC in Gram-Negative Bacteria (GNB) including *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* gives these pathogens the exceptional ability to resist the antimicrobial onslaught of some potent antibiotics[3]. This makes it difficult to select the right therapy for treating infections caused by this resistant GNB because of their multidrug resistance nature. Antibiotic-resistant GNB is among the many bacterial pathogens responsible for most nosocomial and community-acquired infections in the world[3-5]. Resistance of microbes to antimicrobial agents represents a major and serious problem in the health sector, making antibiotic selection for treatment a little more difficult as some of these organisms are multidrug-resistant[6,7]. Multidrug resistance among many organisms has become a big challenge to infectious disease management. It is increasingly being reported in bacteria and is often mediated by genetic mobile elements such as plasmids, transposons and integrons[7,8]. The use of antibiotics for the propagation of farm animals and poultry birds could contribute a great deal in the evolution and spread of resistant bacteria in the community. The undue disposal of abattoir and poultry wastes containing resistant organisms into the environment could also contribute to the spread of resistant GNB. This study investigated the occurrence and distribution of some antibiotic resistance genes in GNB, such as *Escherichia coli*, *Klebsiella* species and *Pseudomonas* spp. from abattoir and poultry milieus in Enugu and Ebonyi States, Nigeria.

MATERIALS AND METHODS

Study area: This study was carried out in the Department of Applied Microbiology Laboratory, Faculty of Science, Ebonyi State University, Nigeria from March, 2018-July, 2019.

Ethical approval: Ethical clearance for this study was obtained from the local ethics committee of Ebonyi State University, Abakaliki, Nigeria. All samples were processed and handled according to all relevant national and international regulations. The research was carried out in collaboration with the Ministry of Agriculture and Animal Breeding, Ebonyi State, Nigeria.

Sampling collection and bacteriological analysis: Non-duplicate abattoir (n = 150) and poultry (n = 300) swab samples were collected from a various abattoir and poultry farms in Enugu and Ebonyi States in Nigeria. All samples were bacteriologically analyzed using standard microbiology techniques including culture, microscopy, biochemical testing, as well as morphological and colonial features of the bacteria on selective culture media[9].

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was carried out using antibiotic discs namely; aztreonam (30 µg), ceftazidime (30 µg), amoxicillin-clavulanic acid (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), imipenem (10 µg), mupirocin (300 µg), sulfamethoxazole-trimethoprim (30 µg), doxycycline (30 µg), colistin sulphate (10 µg), polymyxin B (300 µg), gentamicin (10 µg) and ciprofloxacin (5 µg) as per the guidelines of Clinical Laboratory Standards Institute (CLSI) and according to a previous methodology[10]. Overnight culture of the test *E. coli*, *Klebsiella* and *Pseudomonas* species (adjusted to 0.5 McFarland turbidity standards) were aseptically inoculated on Mueller-Hinton (MH) agar plate(s) and incubated at 37°C for 18-24 hrs. Antibiotic susceptibility results were recorded and interpreted as resistant, intermediate and susceptible according to the CLSI guidelines[10].

Phenotypic detection of ESBL and AmpC enzymes: The presence of ESBL and AmpC enzymes in the test bacteria was phenotypically determined using a double disk synergy test (for ESBL) and disk approximation test (for AmpC enzymes) according to previously described methodology[11]. AmpC enzyme production was phenotypically confirmed when isolates showed obvious blunting or flattening of the zones of inhibition between ceftazidime (CAZ), imipenem or cefotaxime (CTX) discs adjacent to the cefoxitin disc used. A $\geq$5 mm increase in inhibition zone diameter for either of the

Table 1: Primers used for PCR analysis of different antibiotic resistance genes

Primer name	Primer sequence direction	Amplicon size (bp)
DHA-M F	AACTTTCACAGGTGTGCTGGGT	445
DHA-M R	CCGTACGCATACTGGCTTTGC	
FOX-1 F	AACATGGGGTATCAGGGAGATG	747
FOX-1 R	CAAAGCGCGTAACCGGATTGG	
bla_{TEM} F	ATGAGTATTCAACATTTCCG	850
bla_{TEM} R	CCAATGCTTAATCAGTGAGC	
bla_{SHV} F	CTTTACTCGCTTTATCG	445
bla_{SHV} R	TCCCGCAGATAAATCACCA	
bla_{CTX-M} F	CCCATGGTTAAAAAATCACTG	747
bla_{CTX-M} R	CCGTTTCCGCTATTACAAAC	

cephalosporins (CAZ and CTX) was tested in combination with amoxicillin-clavulanic acid (20/10 µg) compared to its zone when tested alone confirms ESBL production phenotypically.

DNA extraction from bacterial isolates: Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ kit according to the manufacturer's protocol. After this, aliquot DNA extracted was stored at -20°C for further analysis. The quality of extracted DNA was assessed and confirmed using 1% agarose gel electrophoresis[12]. Purified DNA was quantified using the NanoDrop spectrophotometer (Thermo Scientific, USA).

PCR screening for ESBL and AmpC resistance genes: All bacterial isolates that showed positive results after phenotypic screening for ESBL and AmpC enzymes were assessed for the presence of ESBL and AmpC genes by PCR according to procedures described by Kolar *et al.*[13]. Primers and targeted amplicon sizes are shown in Table 1. Validation of all primers was done before PCR. The PCR mastermix (50 µL) comprised 5×GoTaq (10 µL), 25 mM $MgCl_2$ (3 µL), dNTPs (10 mM) 1 µL, forward primer (1 µL), reverse primer (1 µL), 10 pmol 1 µL, DNA Taq (1000 U) 25 µL, Ultrapure Water 8 µL. The PCR was performed as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 96°C for 30 sec, the annealing temperature of 58°C for 90 sec, elongation at 72°C for 60 sec; and a final 10 min extension step at 72°C.

RESULTS

Table 2 shows the distribution of bacteria isolated from the various samples bacteriologically analyzed in this study. A total of 42 (45.65%) *E. coli* and 8 (100%) *Klebsiella* spp. isolates were confirmed to be ESBL-producers from poultry samples. On the other hand, a total of 5 (100%) *P. aeruginosa* isolates were phenotypically confirmed as ESBL producers.

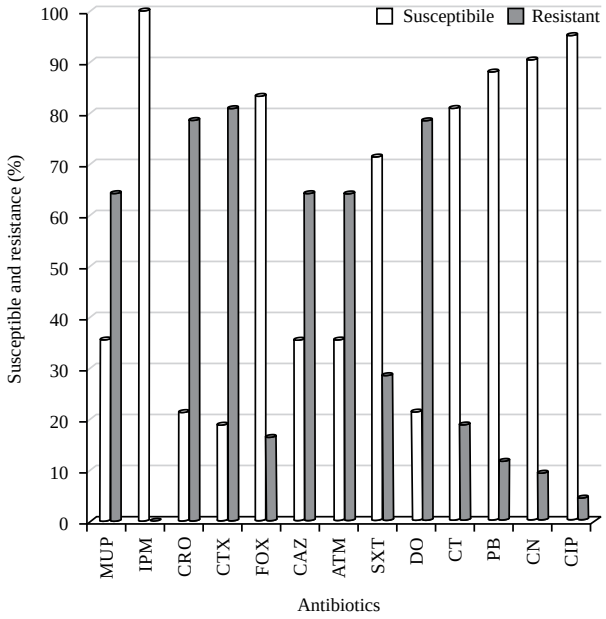

Fig. 1: Susceptibility and resistance profile of the AmpC and ESBL-producing isolates

ATM: Aztreonam, CAZ: Ceftazidime, CRO: Ceftriaxone, CTX: Cefotaxime, FOX: Cefoxitin, IPM: Imipenem, MUP: Mupirocin, SXT: Sulfamethoxazole-trimethoprim, DO: Doxycycline, CT: Colistin sulphate, PB: Polymyxin B, CN: Gentamicin and CIP: Ciprofloxacin

In contrast, 50 (54.35) *E. coli* were confirmed to be ESBL-producers from abattoir samples while *Klebsiella pneumoniae* and *P. aeruginosa* were not isolated. In this study, AmpC enzyme production was phenotypically detected in 33% *E. coli* isolates, 85% *Klebsiella* spp. and 5% *P. aeruginosa* isolates. ESBL-producing *Escherichia coli* isolates from poultry samples were susceptible to a handful of the tested antibiotics including imipenem [42 (100.00%)], ciprofloxacin [40 (95.24%)], gentamicin [38 (90.48%)], polymyxin B [37 (88.10%)], cefoxitin [35 (83.33%)], colistin sulphate [34 (80.95%)], sulfamethoxazole- trimethoprim [30 (71.43%)], ceftazidime [15 (35.71%)] and mupirocin [15 (35.71%)]. However, isolates showed reduced susceptibility to cefotaxime, mupirocin, ceftazidime, aztreonam, cefoxitin, polymyxin B, gentamicin and ciprofloxacin (Fig. 1).

ESBL gene phenotypes including CTX-M, SHV-1 and TEM genes were detected by PCR in the test Gram-negative isolates (Fig. 2). Overall, the CTX-M ESBL genes were most prevalent in the test isolates-indicating a high level of resistance to the cefotaxime antibiotic in the third generation cephalosporin family. On the other hand, the genes that mediate AmpC enzymes in the test Gram-negative bacteria was also detected by PCR, with the DHA-M gene being the most prevalent AmpC gene detected (Fig. 3).

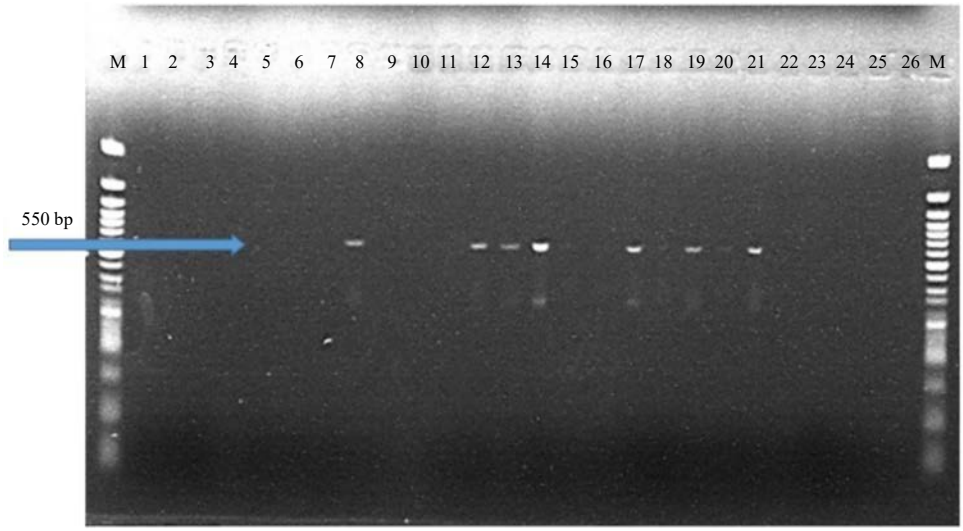

Fig. 2: Electrophoretogram showing PCR detection of CTX-M-ESBL gene in the gram-negative bacterial isolates
Lane M is the DNA marker/ladder. Lanes 8, 12-14, 17, 19 and 21 are lanes showing amplified DNA products-indicative of CTX-M genes or genotypes. Lane 1-7, 9, 10, 15, 16, 18, 20 and 22-25 are lanes showing isolates without amplified DNA products. Lane 26 is the negative control which contains nuclease free water

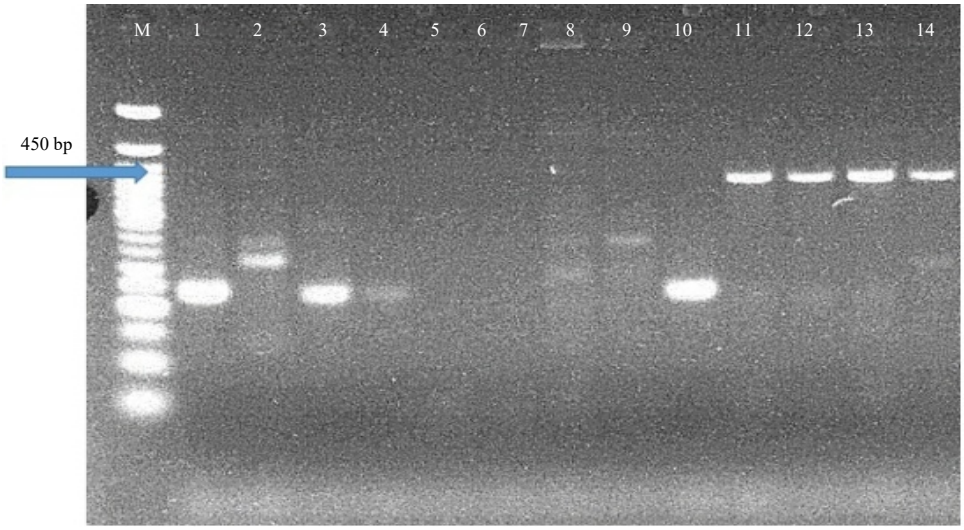

Fig. 3: Electrophoretogram showing PCR detection of DHA-M AmpC gene in the Gram-negative bacterial isolates
Lane M is the DNA marker/ladder, Lanes 11-14 are lanes showing amplified DNA products-indicative of DHA-M genes or genotypes. Lane 5, 6 and 8 are lanes showing isolates without amplified DNA products. Lane 7 is the negative control which contains nuclease free water

Table 2: Distribution of isolated gram-negative bacteria

Sample source	*Escherichia coli*		*Klebsiella* species		*Pseudomonas aeruginosa*	
	Number	Percentage	Number	Percentage	Number	Percentage
Poultry	42	45.65	8	100	5	100
Abattoir	50	54.35	-		-	

DISCUSSION

The global antibiotic resistance threat posed by ESBL and AmpC-producing Gram-Negative Bacteria (GNB), is a public health menace that rolls back the gains of 'One Health' and thus renders some available antibiotics inefficacious for clinical use. This study investigated the occurrence and distribution of some resistance genes in Gram-Negative Bacteria (GNB) such

as *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas* spp. from abattoir and poultry in Enugu and Ebonyi State. These organisms pose a public health risk because of their ability to act as a reservoir for the transmission of antibiotic resistance genes ARGs in both human and animal populations. In this study, ESBL production was phenotypically confirmed in 42 *E. coli*, 8 *Klebsiella* spp. and 5 *P. aeruginosa* isolates. AmpC enzyme production in the test GNB was also observed in more than 50% of the tested organisms in this study. The production of ESBL by the test Gram-negative bacteria in this study was unprecedented and accounts for the probable irrational use of antibiotics for the propagation of animals and poultry birds in this part of the world. This corroborates previous studies conducted in the southwest and southeast Nigeria in which ESBL and AmpC enzyme production was reported at a rate lower than what we reported in this study[11,14,15]. In India and Iran, the prevalence of AmpC was reported to be 12.5 and 37.2%, respectively[16,17] and this result is lower than that reported in our study. The actual prevalence of ESBL and AmpC enzyme-producing Gram-negative bacteria in Nigeria, especially from abattoir and poultry samples is not known. This could probably be due to limited surveillance studies on such multidrug resistance organisms in this region, but studies like ours gives impetus to the growth and spreading nature of these unique class of multidrug-resistant microbes that could impact our healthcare system because of their ability to be transmitted in human population via the food chain. Our results also show that ESBL and AmpC-producing bacteria were multiply-resistant to the tested antibiotic classes used, especially to the cephalosporins, antimetabolites, aminoglycosides, fluoroquinolones and aztreonam. Strikingly, all the AmpC and ESBL-positive bacteria were markedly found to show complete susceptibility to carbapenems, such as imipenem which is an important antibiotic used clinically for the treatment and management of microbial infections including those caused by multidrug-resistant bacteria such as ESBL and AmpC positive organisms[11,18-20]. Gram-negative bacteria producing ESBL and AmpC enzymes have been previously reported to show resistance to both beta-lactam and non-beta-lactam antibiotics as reported in this study[15,16]. The emergence and transmission of bacteria harboring genes for the production of AmpC and ESBL enzymes portend grave danger to the healthcare sector because it limits the options for antimicrobial therapy targeted at infections caused by the pathogens. In this study, we reported the prevalence and distribution of AmpC and ESBL genes amongst the tested GNB investigated. The AmpC resistance genes detected by PCR include ACC-M, MOX-M and DHA-M genes while the ESBL genes that were detected by PCR included CTX-M, SHV-1 and TEM genes. While the DHA-M gene was the most prevalent AmpC gene detected by PCR, the CTX-M gene was the most prevalent ESBL gene detected in this study. CTX-M and DHA-M genes have been reported to cause an increase in the treatment failure of some bacterial infections; including complicated and uncomplicated UTIs due to ESBL and AmpC enzyme production in some priority resistance pathogens[17,21,22]. The levels of ESBL and AmpC enzyme producers as observed in our study are a major threat to infection control, treatment and management because they contribute to treatment failures as reported in this study. ESBL-producing organisms are known to contain plasmids harboring genes that encode resistance phenotypes to a wide variety of antibiotics[1,8,18]. Studies give impetus to the threat posed by antibiotic resistance which has the power to usher the world into a post-antibiotic era if nothing is done to contain it. We, therefore, recommend strict surveillance measures to monitor the emergence and community spread of antibiotic-resistant bacteria in abattoirs and poultry farms.

CONCLUSION

This study reported the emergence and spread of some priority resistance bacteria; including *E. coli*, *Klebsiella* species and *P. aeruginosa* that produce AmpC- and ESBL enzymes which confers on pathogens the ability to resist the antimicrobial onslaught of some available antibiotics. The presence of AmpC and ESBL-producing bacteria in poultry and abattoir milieus in this study is worrisome because they could serve as reservoirs for the transmission of antibiotic resistance genes (ARGs) in the human population.

SIGNIFICANCE STATEMENT

This study has shown that multidrug-resistant ESBL and AmpC-producing Gram-negative bacteria are present in the food chain in South-Eastern Nigeria. Interestingly, our study showed that bacterial isolates exhibited a broad range of resistances to cephalosporins, antimetabolites, aminoglycosides, fluoroquinolones and aztreonam while displaying complete susceptibility to carbapenems, such as imipenem. Our study will provide a baseline template for future surveillance studies on the prevalence of multidrug-resistant bacteria in food sources in Nigeria so as to help in combating the increasing menace of antibiotic resistance which is a serious public health problem.

REFERENCES

1. Jacoby, G.A. and L.S. Munoz-Price, 2005. Mechanisms of disease: The new beta-lactamases. N. Engl. J. Med., 352: 380-391.
2. Bush, K., G.A. Jacoby and A.A. Medeiros, 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother., 39: 1211-1233.
3. Zhang, C.H., Y.L. Liu and J.H. Wang, 2010. Detection of ESBLs and antimicrobial susceptibility of *Escherichia coli* isolated in Henan, China. J. Anim. Vet. Adv., 9: 2030-2034.
4. Zurfluh, K., H. Hächler, M. Nüesch-Inderbinen and R. Stephan, 2013. Characteristics of extended-spectrum beta-lactamase- and carbapenemase-producing *Enterobacteriaceae* isolates from rivers and lakes in Switzerland. Appl. Environ. Microbiol., 79: 3021-3026.
5. Zhu, B., P. Zhang, Z. Huang, H.Q. Yan, A.H. Wu, G.W. Zhang and Q. Mao, 2013. Study on drug resistance of *Pseudomonas aeruginosa* plasmid-mediated AmpC β-lactamase. Mol. Med. Rep., 7: 664-668.
6. Fernandez, M., S. Conde, J. Torre, C. Molina-Santiago, J.L. Ramos and E. Duque, 2012. Mechanisms of resistance to chloramphenicol in *Pseudomonas putida* KT2440. Antimicrob. Agents Chemother., 56: 1001-1009.
7. Franco, M.R., H.H. Caiaffa-Filho, M.N. Burattini and F. Rossi, 2010. Metallo-beta-lactamases among imipenem-resistant *Pseudomonas aeruginosa* in a Brazilian university hospital. Clinics, 65: 825-829.
8. Bush, K. and G.A. Jacoby, 2010. Updated functional classification of β-lactamases. Antimicrob. Agents Chemother., 54: 969-976.
9. Cheesbrough, M., 2000. District Laboratory Practice in Tropical Countries. 1st Edn., Press Syndicate of the University of Cambridge, Cambridge UK., pp: 132-143.
10. Clinical Laboratory Standard Institute (CLSI), 2011. Performance standards for antimicrobial disk susceptibility test. Fifteenth informational supplement, CLSI document M100-S15. Wayne, PA. USA.
11. Chika, E., I. Ifeanyichukwu, U. Malachy, O. Benigna and E.C. Adaora *et al*., 2016. Phenotypic detection of AmpC enzymes and antimicrobial susceptibility of *Klebsiella* spp. isolated from abattoir. Int. J. Appl. Microbiol. Biotechnol. Res., 4: 117-121.
12. Iroha, I.R., E.S. Amadi, A.E. Oji, A.C. Nwuzo and P.C. Ejike-Ugwu, 2010. Detection of plasmid borne extended spectrum beta lactamase enzymes from blood and urine isolates of gram-negative bacteria from a University Teaching Hospital in Nigeria. Curr. Res. Bacteriol., 3: 77-83.
13. Kolar, M., J. Bardon, M. Chroma, K. Hricova, T. Stosova, P. Sauer and D. Koukalova, 2010. ESBL and AmpC beta-lactamase-producing *Enterobacteriaceae* in poultry in the Czech Republic. Vet. Med., 55: 119-124.
14. Iroha, I.R., M.U. Adikwu, E.S. Amadi, I. Aibinu and C.O. Esimone, 2008. Characterization of extended spectrum β-lactamase producing *E. coli* from secondary and tertiary hospital in South Eastern Nigeria. Res. J. Microb., 3: 514-519.
15. Motayo, B.O., P.A. Akinduti, F.A. Adeyakinu, P.O. Okerentugba and J.C. Nwanze *et al*., 2013. Antibiogram and plasmid profiling of carbapenemase and extended spectrum Beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* in Abeokuta, South western, Nigeria. Afr. Health Sci., 13: 1091-1097.
16. Yilmaz, N.O., N. Agus, E. Bozcal, O. Oner and A. Uzel, 2013. Detection of plasmid-mediated AmpC β-lactamase in *Escherichia coli* and *Klebsiella pneumoniae*. Indian J. Med. Microbiol., 31: 53-59.
17. Mansouri, S., D.K. Neyestanaka, M. Shokoohi, S. Halimi, R. Beigverdi, F. Rezagholezadeh and A. Hashemi, 2014. Characterization of AmpC, CTX-M and MBLs types of β-Lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* producing Extended Spectrum β-lactamases in Kerman, Iran. Jundishapur J. Microbiol., Vol. 7, No. 2. 10.5812/jjm.8756.
18. Jacoby, G.A., 2009. AmpC β-lactamases. Clin. Microbiol. Rev., 22: 161-182.
19. Walsh, T.R., M.A. Toleman, L. Poirel and P. Nordmann, 2005. Metallo-β-lactamse: The quiet before the storm. Clin. Microbiol. Rev., 18: 306-325.
20. Thomson, K.S., 2010. Extended-spectrum-β-lactamase, AmpC and carbapenemase issues. J. Clin. Microbiol., 48: 1019-1025.
21. Kanu, A.M., N. Mgbajiaka and N. Abadom, 2016. Prevalence of urinary tract infection among HIV patients in Aba, Nigeria. Int. J. Infect. Dis., Vol. 45. 10.1016/j.ijid.2016.02.517.
22. Manchanda, V. and N.P. Singh, 2003. Occurrence and detection of AmpC β-lactamases among gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. J. Antimicrob. Chemother., 51: 415-418.