ORIGINAL RESEARCH ARTICLE

First Report Occurrence of *CIT* and *DHA*AmpCβ-lactamase gene in *Escherichia coli* and *Klebsiellapnuemoniae* from clinical sample in south eastern, Nigeria.

ABSTRACT

Background and Objectives:

AmpC*β*-lactamases have gained importance over the years, since their discovery as one of the enzymes responsible for antimicrobial resistance in Gram-negative bacilli especially in *Escherichia coli* and *Klebsiellapnuemoniae,* it has led to a significant challenge to patient care. Hence, this study was designed to assess the occurrence of *CIT* and *DHA*AmpCβ-lactamase gene in *Escherichia coli* and *Klebsiellapnuemoniae* from clinical sample in south eastern, Nigeria

Methodology:

This cross-sectional study was conducted over an 8-month period on sixteen (16) consecutive non-repetitive isolates of *Escherichia coli* and *Klebsiellapnuemoniae* obtained from the diagnostic laboratories and medical unit of Alex Ekweume Federal University Teaching Hospital in Abakaliki, Nigeria. Isolates were accurately identified through routine microbiological diagnostic tests. The bacteria were first tested for cefoxitin resistance using a disc diffusion test, followed by phenotypic confirmation of AmpC production using phenyl boronic acid assays. *Escherichia coli* and *Klebsiellapneumoniae*strains were further screen for AmpCbetalactamase*CIT* and *DHA* genotypeby polymerase chain reactions

Result: Of the sixteen (16) confirmed phenotypic AmpC β-lactamase producing bacteria, 100 % of the following AmpC β-lactamase genes (*bla*DHA, *bla*CIT) were detected in *E. coli* from urine and wound samples of both male and female patients**.** The overall proportion of AmpC β-lactamases gene in *Klebsiellapneumoniae*were *blaDHA*(100 %) and *blaCIT*(100 %), in both male and female.

Conclusion: This study indicate the occurrence of *CIT* and *DHA*AmpC genotype. The AmpC β-lactamases found in this study are clinically relevant β-lactamases because they can confer a MDR phenotype. Thus awareness of the occurrence of AmpC β-lactamase-producing bacteria could be very valuable for achieving more accurate epidemiological results, as well as controlling their spread while surveillance is needed to follow any further emergence of other AmpC β-lactamase genotype

Keyword: *AmpC*β-lactamases, *Escherichia coli*, *Klebsiellapneumoniae*, *CIT*,*DHA*

1. INTRODUCTION

Escherichia coli and *Klebsiellapnuemoniae*are members of the*Enterobacteriaceae* family. This family's two critical genera are associated with both opportunistic nosocomial pathogens. These two genera, especially *E.coli*and *Klebsiellapneumoniae*, are opportunistic pathogens that cause septicemia, bacteremia, infantile enteritis, meningitis, and urinary tract infections of soft tissues [1, 2]. These human pathogens are responsible for infections and diseases in hospitalized patients with immunodeficiency and underlying conditions such as diabetes mellitus and chronic pulmonary disorders [1, 2]. However, the widespread use of antibiotics poses a selective pressure leading to the selection of resistant bacteria and has dramatically increased over the years endangering the lives of many patients. A recent systematic review identified an increase in the worldwide prevalence of AmpCbetalactamase in the Gram negative organisms *Escherichia coli* and *Klebsiellapneumoniae* over the past ten years [3].Some *Escherichia coli* and *Klebsiellapnuemoniae*are capable of producing enzymes called AmpCβ-lactamases, characterized by their ability to inactivate cephamycins in addition to other extended-spectrum cephalosporins and being resistant to clavulanic acid [4]. Infections caused byAmpC-positive bacteria are of particular clinical and epidemiological importance and cause higher patient morbidity and mortality [2, 5, 6]. According previous reports in Brahain, India, Pakistan, Korea, and Spain, the majority of the strains with AmpC genotype are generally multidrug resistant [7, 8, 9, 10]. AmpCβ-lactamase gene are encoded by chromosomes or plasmids and affect a wide range of β-lactam drugs. Based on the sequence similarities with species-specific AmpC enzymes, plasmid AmpC variants are classified into

five evolutionary groups: the CIT variants (CMY-2 types) originating in *Citrobacterfreundii*, the *Enterobacter*sp. EBC variants (ACT-1 type, MIR-1), the *Morganellamorganii*DHA variants, the *Hafniaalvei*ACC variants, and the *Aeromonas*sp. FOX and MOX variants, one of which is widely expressed in each geographical region [2, 6, 10, 11, 12] but the knowledge of someAmpCbetalactamasegeneotype circulating this area remain unknown. Among these, *ACC, FOX, MOX, DHA, CMY, CIT*, and *EBC* genotypes are most commonly reported elsewhere [2, 610]. To date, no clinical research on the occurrence of AmpCβ-lactamases genotype have been conducted in Abakaliki, South eastern, Nigeria. Accurate detection of AmpCgenotype (*CIT* and *DHA*) is not only critical for proper health management of patients suffering from *Escherichia coli* and *Klebsiellapnuemoniae*infections but also useful for epidemiological analyses of the geographical distribution of AmpC genes.

2. MATERIALS AND METHOD

2.1 Bacterial Isolation

The study received Ethical review board approval (number SMOH/ERC/042/21) from the ethics committee of the Ebonyi State Ministry of Health, Abakaliki and conducted in accordance with the Declaration of Helsinki [13, 14].
This cross-sectional study was performed during period of 8-months on sixteen (16) consecutive non-repetitive isolates of *Escherichia coli* and *Klebsiellapnuemoniae*obtained from diagnostic laboratories and medical unit ofAlex Ekweume Federal University Teaching Hospital, Abakaliki in southeastern Nigeria and it located in 6.32^{o}N latitude and 8.12^{o}E and longitude and is situated at an elevation of 117 meters above sea level. Isolates were accurately identified through routine microbiological diagnostic tests [15, 16]. The confirmed isolates were stored in a brain-heart infusion broth (Merck Co. Germany) containing 20% glycerol at −70°C until further test [15].

2.2 Screening and Confirmatory Testing of AmpC Production

All isolates were first screened for the probable production of AmpC β-lactamases by placing a cefoxitin disk (30 µg) on Mueller-Hinton agar (Merck Co., Germany) [17]. Isolates that conferred an inhibition zone diameter of <18 mm were considered potential producers of AmpC and subjected to the confirmatory phenotypic test. Screening-positive AmpC producers were confirmed by an inhibitor-based method on a disk containing boronic acid [18]. Briefly, a lawn culture of the tested isolates was made on a Mueller-Hinton agar plate using 0.5 McFarland solutions. Two disks of cefoxitin (30 µg) with and without phenylboronic acid (400 µg) was placed onto the agar surface and the results were interpreted. If the growth inhibition zone around the antibiotic with phenylboronic acid was 5 mm or greater when compared to the disk containing only cefoxitin, the isolate was considered an AmpC producer [17, 18].

2.3 Bacterial DNA extraction and polymerase chain reaction

The bacterial DNA of phenotypic confirmed AmpCbetalactamse producing *E. coli and K. pneumoniae*were extracted from pure overnight cultures using the ZR fungal/bacterial DNA MiniPrep kit [14, 19]. The AmpC-betalactamas encoding genes (CIT, and DHA) were amplified individually on a SimpliAmp™ thermal cycler (Applied Biosystems, Foster City, CA) using the specific primers (Invitrogen,U.S. A)for*CIT*gene F-TGGCCAGAACTGACAGGCAAA; R- TTTCTCCTGAACGTGGCTGGC; *DHA* gene; F-AACTTTCACAGGTGTGCTGGGT; R- CCGTACGCATACTGGCTTTGC [20].The amplification reaction was performed in a final volume of 20 µL containing Master Mix (Bioneer, South Korea), primers at concentrations of 10 pM, 50–100 ng of extracted DNA templates, and ddH2O. The PCR conditions for the amplifications were as follows, 5 min at 95°C for the initial denaturation step; 30 cycles of 30 sec at 95°C for DNA denaturation, 30 sec for primer annealing). The temperature depended on the sequences of primers (primer extension at 72 °C for 1 min and a final extension of 5 min at 72°C. The PCR products were separated on 1.5% agarose gel prepared in 1X TBE (Tris/Boric/EDTA) buffer and visualized under UV transilluminator using a gel documentation system.

3. RESULT

3.1 Occurrence of AmpC β-lactamase genes in isolates of *E. coli and K. pneumoniae*

Of the sixteen (16) confirmed phenotypic AmpC β-lactamase producing bacteria, 100 % of the following AmpC β-lactamase genes (*bla*DHA, *bla*CIT) were detected in *E. coli* from urine and wound samples of both male and female patients as shown in Table 1**.** The overall proportion of AmpC β-lactamases gene were *blaDHA*(100 %) and *blaCIT*(100 %), in both male and female as shown in Table 2.

Table 1: Occurrence of AmpC β-lactamase genes in isolates of *E. coli*

Clinical Sample	Gender	Bacteria coding	*bla**DHA* (%)	*bla**CIT* (%)

Urine	Male	E1	1(12.5)	1(12.5)
		E2	1(12.5)	1(12.5)
	Female	E3	1(12.5)	1(12.5)
		E4	1(12.5)	1(12.5)
Wound swab	Male	E5	1(12.5)	1(12.5)
		E6	1(12.5)	1(12.5)
	Female	E7	1(12.5)	1(12.5)
		E8	1(12.5)	1(12.5)
Total		**(n=8)**	**8(100)**	**8(100)**

Table 2: Occurence of AmpC β-lactamase genes in *K. pneumoniae*isolates

Clinical Sample	**Gender**	**Bacteria coding**	***DHA* (%)**	***blaCIT* (%)**
Urine	Male	K9	1(12.5)	1(12.5)
		K10	1(12.5)	1(12.5)
	Female	K11	1(12.5)	1(12.5)
		K12	1(12.5)	1(12.5)
Wound swab	Male	K13	1(12.5)	1(12.5)
		K14	1(12.5)	1(12.5)
	Female	K15	1(12.5)	1(12.5)
		K16	1(12.5)	1(12.5)
Total		**(n=8)**	**8(100)**	**8(100)**

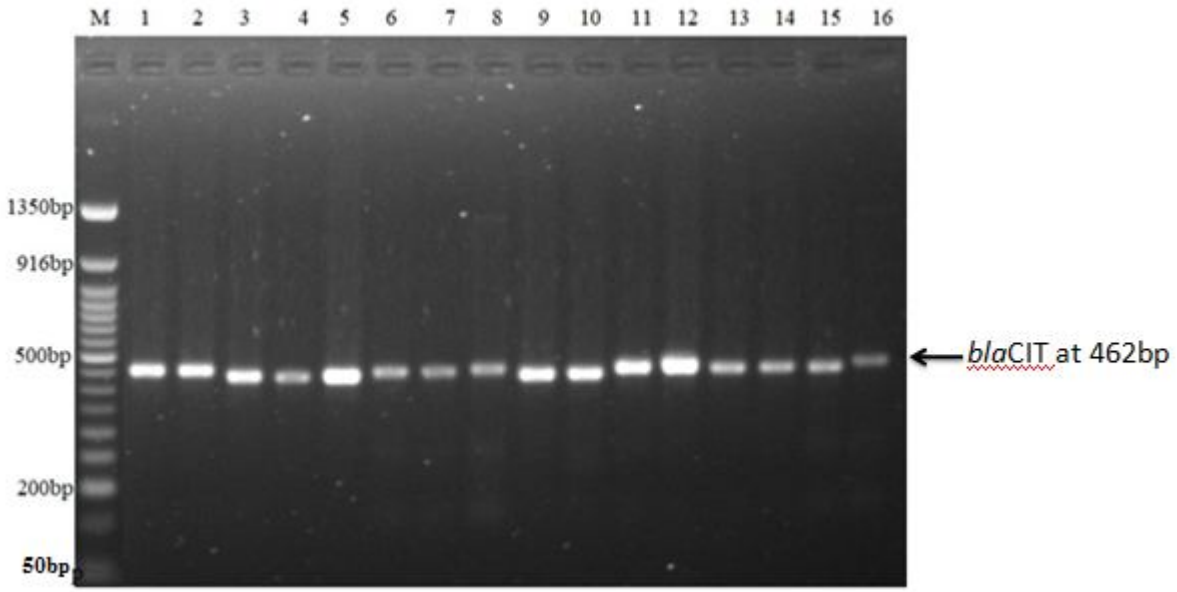

Plate 1: Gel image amplification of *bla*CIT at about 462bp. A 50bp ladder was used to estimate the base pair size of the amplicons. Lane M – 50bp Molecular marker, Lane 1 – 8 =*E. coli*, Lane 9 – 16 = *K. pneumoniae* isolates.

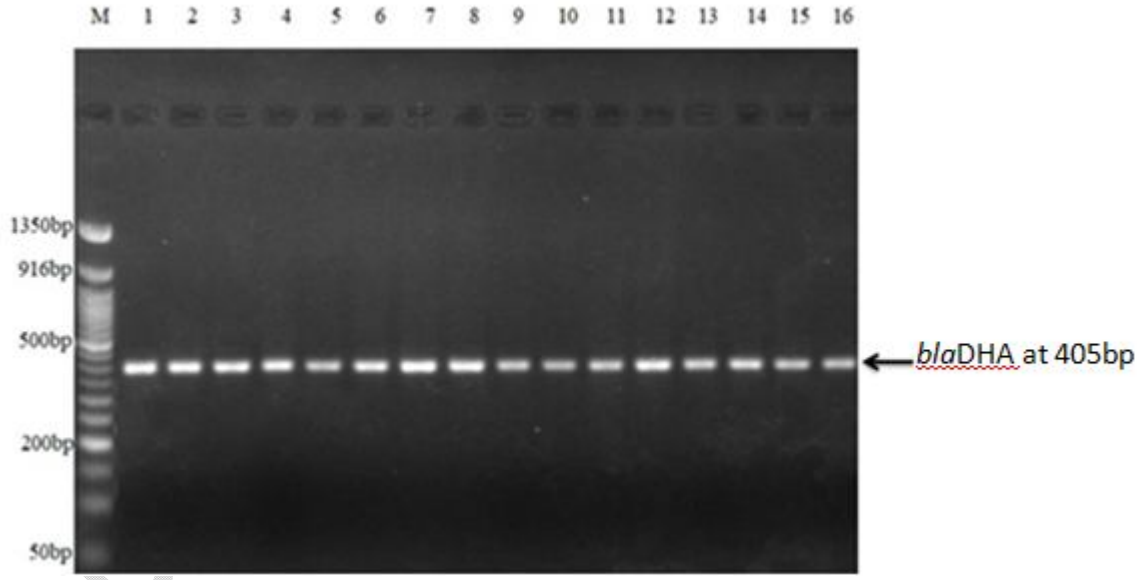

Plate 2: Gel image amplification of *bla*DHA at about 302bp. A 50bp ladder was used to estimate the base pair size of the amplicons. Lane M – 50bp Molecular marker, Lane 1 – 8 =*E. coli*, Lane 9 – 16 = *K. pneumoniae* isolates.

4. DISCUSSIONS

AmpCβ-lactamases was identified by PCR which revealed the predominance ofbla_{DHA}(100 %), and bla_{CIT}(100 %). Our findings reiterate with report from other studies; Malek*iet al.* [21] in Ilam (West of Iran) reported *CIT* and DHA as the most frequent AmpC genes in *E. coli*isolates. Ghanavati*et al.*[22] in Tehran (North of Iran) also reported the clusters of *CIT*, and *DHA* genes as the most abundant genes in *Klebsiella* isolates, respectively. In Bahrain plasmid-derived AmpC β-lactamase among clinical strains of *Klebsiellapneumoniae* and *E. coli* 10.3% and 31% of cefoxitin-resistant isolates harbored the *DHA* and *CIT* gene, respectively [10]. Geographic diversity in the molecular subtypes of AmpC genes has been discovered through studies conducted in various parts of the world. In this regard, the CIT AmpC subtype has been widely identified in the United States and Canada [23]. Woodford *et al.* [24] also described the prevalence of *CIT*positive, AmpC producing *E. coli* in the United Kingdom and Ireland.

In a study by Mohamudha*et al.* [25], *DHA* was more common in both *Klebsiella* species and *E. coli* isolates (46.7% and 38%, respectively). DHA-1 is a plasmid-mediated AmpC-lactamase derived from *M. Morganii's* chromosomal AmpC gene [26]. In a study conducted by Pai*et al.* [27] and Molande*t al.*[28], it was discovered that mortality of patients infected with organisms that produce DHA-1 was higher than that of patients infected with organisms that produce CMY-1, raising concerns about the spread of this inducible plasmid-mediated AmpC-lactamase.

In another report, all *E. coli* isolates were positive for the *CIT* family [29, 30]. In this regard, *blaDHA*, *blaCIT*, type of AmpC has been widely detected in this study showing rapid plasmid expression and dissemination of the gene in the area in recent time. Although they seems to be a variation in the occurrence of $bla_{DHA}$and bla_{CIT}reportedin this study. Such discrepancies could be linked to geographical area, number of samples, the species studied, and the periodof study influence the prevalence, and type of acquired*AmpC*s detected. As a result, comparing the prevalence of acquired AmpCs across studies is difficult.

According to the findings, the *CIT* and *DHA* gene appears to be the most important factor in the plasmid dissemination of AmpC producing isolates. The AmpC β-lactamases found in this study are clinically relevant β-lactamases because they can confer a MDR phenotype and also expressed resistance towards β-lactamase inhibitors, broader cephalosporin because resistance can arise in such strains by further mutations, resulting in reduced porin expression.

5. CONCLUSION

The study provides data of first report occurrence of *DHA* and*CIT* types *AmpC*isolates which is alarming. The spread of these AmpC β-lactamases resistance genes within the hospital or across the country via conjugation may become a major public health concern. As a result of identifying *DHA* and*CIT* types of AmpC may assist physicians in prescribing the most appropriate antibiotic, reducing the selective pressure that leads to antibiotic resistance.

LIMITATIONS

The lack of data on the sequencing of AmpC cluster genes was one of the study's limitations. Furthermore, due to a lack of funding, only the presence of *CIT* and *DHA*AmpC genes was investigated, while other AmpC genes, chromosomal hyperproduction or purine loss mutations, were not investigated

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

In compliance with international standard or university standard written ethical approval has been collected and preserved by the author (s).

REFERENCES

1. Kaye KS, Pogue JM. Infections caused by resistant gram-negative bacteria: epidemiology and management. Pharmacotherapy: *J Human Pharm DrugTher*. 2015;35(10):949-962

2. GolshaR, Maryam M, Nazanin R, Mina EZ. Frequency of Beta-Lactamase Antibiotic Resistance Genes in *Escherichia Coli* and *Klebsiellapneumoniae*, Gorgan, Iran. *Ethiop J Health Sci*. 2021;31 (3):663

3. Rodríguez-Guerrero E, Callejas-Rodelas JC, Navarro-Marí JM,Gutiérrez- Fernández J. Systematic review of plasmid AmpC type resistances in *Escherichia coli* and *Klebsiellapneumoniae* and Preliminary Proposal of a Simplified Screening Method for AmpC. *Microorganisms*. 2022; 10:611-612.

4. Singhal S.,Mathur T, Khan S. Evaluation of methods for AmpC*β*-lactamase in Gram-negative Clinical Isolates from Tertiary Care Hospitals. *Indian J Medical Microbiol*. 2005; 23(2):120–124.

5. Livermore DM. Current epidemiology and growing resistance of Gram-negative Pathogens. *Korean J InternalMed*. 2012; 27(2):128–142.

6. Sadeghi H M,Ebrahim-Saraie S, Mojtahedi A. Prevalence of ESBL and AmpC genes in E. coli isolates from urinary tract infections in the north of Iran. *New Microbes and New Infections*. 2021; 45:47-60

7. Martinez‐Martinez L, Conejo MC, Pascual A, Hernandez‐Alles S, Ballesta S, Ramírez De Arellano‐Ramos E, *et al.* Activities of Imipenemand Cephalosporinsagainst Clonally Related Strains of *Escherichia coli* hyperproducing chromosomal beta‐lactamase and showing altered porin profiles. Antimicrob Agents Chemother 2000;44:2534‐6.

8. Park SD, Uh Y, Lee G, Lim K, Kim JB, Jeong SH. Prevalence and resistance patterns of extended-spectrum and AmpC β-lactamase in *Escherichia coli*, *Klebsiellapneumoniae*, *Proteusmirabilis*, and *Salmonella*serovar*stanley* in a Korean Tertiary Hospital. *J ClinMicrobiol*. 2010; 118(10):801–808.

9. Jameel NA, Ejaz H, Zafar A, Amin H. Multidrug Resistant AmpC β-lactamase Producing *Escherichia coli* Isolated from a Paediatric Hospital. *Pak J Med Sci.* 2014; 30(1):181-184

10. Joji RM, Al-Mahameed AE, Al Jishi T, Fatani DI, Saeed NK, Jaradat A, *et al.* Molecular detection of plasmid-derived AmpC β-lactamase among clinical strains of *Enterobacteriaceae*in Bahrain. Ann Thorac Med 2021;16:287-93.

11. Ejikeugwu, C., Esimone, C., Iroha, I. R and Adikwu, M (2018). First Detection of FOX-1 AmpC β-lactamase Gene Expression Among*Escherichia coli* Isolated from Abattoir Samples in Abakaliki, Nigeria. *Oman Medical Journal*, **33**(3):243-249.
12. Hanson ND. AmpC beta‐lactamases: what do we need to know for the future? J AntimicrobChemother 2003;52:2‐4.
13. World Medical Association (WMA) Declaration of Helsinki. Ethical principles for Medical Research involving Human Subjects. Note of Clarification on paragraph 30 by the WMA General Assembly, Tokyo. 2004;9-10.
14. Peter IU, Emelda NC, Chukwu EB, Ngwu JN, Uzoeto HO, Moneth EC Stella AO, Edemekong CI, Uzoamaka EP, Nwuzo AC, Iroha IR. Molecular Detection of Bone Sialoprotein-Binding Protein (*bbp*) Genes among Clinical Isolates of Methicillin Resistant *Staphylococcus aureus*from Hospitalized Orthopedic Wound Patients. *Asian J Orthopaedic Res.* 2022, 8(3):1-9.
15. Iroha IR, Orji JO, Onwa NC, Nwuzo AC, Okonkwo EC, Ibiam EO, Nwachi AC, Afuikwa FN, Agah VM, Ejikeugwu EPC, Agumah NB, Moses IB, Ugbo E, Ukpai EG, Nwakaeze E A, Oke B, Ogbu L and Nwunna E. Microbiology Practical Handbook. (Editor; Ogbu. O), 1st Edition. Charlieteximage Africa (CiAfrica Press), 2019; Pp:344.

16. Edemekong, CI, Iroha IR, Thompson, MD, Okolo, IO., Uzoeto HO, Ngwu JN, Mohammed ID,Chukwu EB, Nwuzo AC, Okike BM, Okolie SO, Peter IU. Phenotypic Characterization and Antibiogram of Non-Oral Bacteria Isolates from Patients Attending Dental Clinic at Federal College of Dental Technology and Therapy Medical Center Enugu. *Int J Pathog Res.*2022;11(2): 7-19.

17. Polsfuss S, Bloemberg GV, Giger J, Meyer V,Böttger EC, Hombach M. Practical Approach for Reliable Detection of AmpC beta-lactamase-producing Enterobacteriaceae. *J ClinMicrobiol.* 2011; 49(8):2798-2803.
18. Coudron PE. Inhibitor-based methods for detection of plasmidmediatedAmpC beta-lactamases in Klebsiella spp., *Escherichia coli*, and *Proteus mirabilis*. *J ClinMicrobiol* 2005;43:4163−7.

19. Edemekong CI, Uzoeto HO, Mbong EO, Ikusika BA, Didiugwu, CM, Ngwu JN, NseAbasi PL, Ntekpe ME, Mohammed ID, John-Onwe BN, Alagba EE, Obodoechi IF, Joseph OV, Ogbonna IP, Ubom IJ, Peter IU (2022). Molecular Characterization and Bioassay of Soil *Actinomycetes* Strains on Multidrug Resistant Bacteria. *IOSR J BiotechnolBiochem*. 2022; (1):6-11.
20. Pérez-Pérez FJ, Hanson ND. Detection of Plasmid-mediated *AmpC* Beta-lactamase genes in Clinical Isolates by using Multiplex PCR. *J ClinMicrobiol*. 2002; 40:2153–62.
21. Maleki A, Khosravi A, Ghafourian S, Pakzad I, Hosseini S, Ramazanzadeh R, et al. High prevalence of AmpC β-Lactamases in clinical isolates of *Escherichia coli* in Ilam, Iran. *Osong Public Health Res Perspectives* 2015;6(3):201–4.

22. Ghanavati R, Darban-Sarokhalil D, Navab-Moghadam F, Kazemian H, Irajian G, Razavi S. First report of coexistence of AmpCbetalactamase genes in *Klebsiellapneumoniae* strains isolated from burn patients. *Actamicrobiologicaetimmunologica Hungarica* 2017;64(4): 455–62.

23. Oliveira C, Amador P, Prudêncio C, Tomaz CT, Tavares‐Ratado P, Fernandes R. ESBL and AmpC‐lactamases in clinical strains of *Escherichia coli* from Serra da Estrela, Portugal. *Medicina (Kaunas)* 2019;55 :272.

24. Woodford N, Reddy S, Fagan EJ, Hill RL, Hopkins KL, Kaufmann ME. Wide geographic spread of diverse acquired AmpC beta‐lactamases among *Escherichia coli* and *Klebsiella*species in the UK and Ireland. *J AntimicrobChemother*; 2007;59:102‐5

25. Mohamudha, PR, Harish BN, Parija SC. Molecular Description of Plasmid-mediated AmpC beta-lactamases among Nosocomial Isolates of *Escherichia coli* and *Klebsiellapneumoniae* from Six Different Hospitals in India. *Indian J Medl Res.* 2012; 135:114–9.

26. Verdet C,Arlet G,Barnaud G,Lagrange PH, Philippon A. A novel integron in *Salmonella enterica*serovarenteritidis, carrying the *bla*(DHA-1) gene and its regulator gene *amp*R, originated from *Morganellamorganii*.*AntimicrobAgents and Chemother*. 2000; 44(1):222–225.

27. Pai H,Kang CI,Byeon JH. Epidemiology and clinical features of bloodstream infections caused by AmpC-type- *β*-lactamase-producing *Klebsiellapneumoniae*. *Antimicrob Agents and Chemother*.2004; 48(10): 3720–3728.
28. Moland ES, Kim SY, Hong SG, Thomson KS. Newer beta-lactamases: clinical and laboratory implications, part I," *ClinMicrobiol Newsletter*. 2008;(30):71–77.

29. Dallal MS, Sabbaghi A, Aghamirzaeie HM, Lari AR, Eshraghian MR, Mehrabad JF. Prevalence of AmpC and SHV Beta-lactamases in Clinical Isolates of *Escherichia coli* from Tehran Hospitals. *Jundishapur J Microbiol.* 2013; 6(2):176-80.

30. Thoms Rodriguez CA, DawsonF, Cameron J, Seah C, Reid M, Melano RG, Gossell-Williams M. Prevalence and distribution of AmpC beta-lactamase producing *Escherichia coli* and *Klebsiellapneumoniae*isolates obtained from urine samples at a tertiary care hospital in the Caribbean. *Front. Cell. Infect. Microbiol*. 2022; 12:1015-633.