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Molecular Characterization of ESBLs Genes among Multi-drug Resistant *Klebsiella* species in Ile-Ife South-West, Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Author OAT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AOO supervised and managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was carried out to determine the presence of ESBLs genes (TEM, CTX-M, SHV, AAC and OXA types of Beta-lactamases) in clinical isolates of *Klebsiella* species producing extended spectrum beta-lactamase.

Extended Spectrum Beta Lactamase (ESBL)-producing *Klebsiella* have increased rapidly and became a major problem in the area of infectious diseases. Nosocomial infections are primarily caused by *Klebsiella* bacteria, which leads to an increase in health care costs and mortality rate.

Methodology: A total of 166 bacteria was isolated. Resistance to different antibiotics was determined using the standard disk diffusion method. Extended spectrum beta-lactamases were screened for using disc diffusion method, confirmatory test was detected by the double disk synergy

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test (DDST) method and polymerase chain reaction (PCR) was used to determine bla*CTX*, bla*OXA*, bla*SHV*, *AAC* and bla*TEM* genes in the ESBLs positive isolates.
Results: bla*TEM* (31%) was the most prevalent and AAC (27%). The rates of CTX-M and SHV genes are 21% and 13% respectively.
Conclusion: Outbreak of isolates producing ESBLs can cause serious problems in the future, regarding the treatment of infections caused by this common pneumonia pathogen.

Keywords: Multi-drug; resistant; ESBLs; Klebsiella species; Ile-Ife.

1. INTRODUCTION

Extended spectrum ß-lactamases (ESBLs) are ß-lactamases that hydrolyze extended spectrum cephalosporins such as cefotaxime and ceftazidime. According to Ambler classification, AmpC-ß lactamases are an important group of class C ß-lactamases that hydrolize penicillins, extended-spectrum cephalosporins, cephamycins and aztreonam, however they can't be inhibited by ß-lactamase inhibitors such as clavulanate, sulbactam and tazobactam, but are inhibited by phenylboronic acid and cloxacillin [1-2]. The production of β-lactamase remains the most important contributing factor to β-lactam resistance [3]. Extended spectrum β-lactamases (ESBLs) have been reported to be common cause of hospital-acquired infections and can have severe clinical implications with a multiple antibiotic resistances [4]. The β-lactamases enzymes, deactivate the molecular antibacterial properties of β-lactam antibiotics by breaking and opening the β-lactam ring. Bacteria harboring these enzymes are usually resistant to β-lactam antibiotics as well as antibiotics from other classes thereby posing a therapeutic challenge to clinicians [5]. ESBLs enzymes are carried in and transferred by bacteria plasmid and are responsible for bacteria resistance to β-lactam antibiotics but are inhibited by β-lactamase inhibitors [6]. Antibiotic selection for infections due to ESBL-producing pathogens is still a clinical challenge. CTX and TEM type of the ß-lactamase enzymes is a ESBLs type that is widely reported in Enterobacteriaceae such as *K. pneumoniae*.

Klebsiella pneumoniae are important causes of different bacterial infections, including, bacteremia, respiratory and urinary tract infections, neonatal meningitis and pneumonia [7-9]. The ß-lactams antibiotics are one of the treatment choices for these bacterial infections [10]. One of the main mechanisms of resistance to ß-lactams antibiotics is via the actions of ß-lactamase enzymes [7]. This enzyme is the predominant ESBLs type in some countries [11]. The CTX enzymes are usually encoded by genes that are carried on the plasmid and have greater activity against cefotaxime than other ceftazidime [12].

2. METHODOLOGY

2.1 Bacterial Strains

In total, 166 *Klebsiella* species were isolated from sputum and throat swab of patient with respiratory tract infection. The isolates were identified based on their cultural, morphological characteristics and reactions to standard biochemical tests. The isolates were further confirmed using Analytical profile index 20E kit (bioMérieux, Inc., France).

2.2 Antimicrobial Susceptibility Testing

The antibiotic resistance pattern of the isolates was determined by using the disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [13]. The antibiotics single disc (Oxoid Ltd, Basingstoke, Hampshire, England) tested were cefotaxime (30 µg), piperacillin (10 µg), augmentin (2 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), ceftriazone, (30 µg), ofloxacin (5 µg), cefixime (5 µg), imipenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg) and nitrofurantoin (300 µg).

2.3 Detection of ESBLs Producing Isolates

ESBLs producing isolates were detected using double disk synergy test (DDST) as a standard disk diffusion assay on Mueller Hinton agar (Himedia, India) [8]. The presence of ESBLs was assayed using the following antibiotic disks: ceftazidime (30 µg), cefotaxime (30 µg) and amoxycillin/clavulanic acid (20/10 µg) (Oxoid Ltd, Basingstoke, Hampshire, England).

Disks containing ceftazidime (30 µg) and cefotaxime (30 µg) alone was placed 25 mm apart, center to center to the amoxycillin/clavulanic acid (20/10 µg) disc on a lawn culture of the test isolate on Mueller-Hinton agar plate. The plates were incubated at 37℃ for 18-24 h. An enhanced zone of inhibition towards the amoxycillin/clavulanic acid (20/10 µg) disc indicated positive ESBL production.

2.4 DNA Extraction and Amplification

The primers used for PCR amplification were blaCTX, SHV, ACC, OXA and bla TEM (Promega, USA) (Table 1). Polymerase Chain Reaction (PCR) amplification reaction was set up in a PCR vial, after adding the master mix, the forward and reverse primers and the extracted DNA. Twenty five microliter (25 µl) of master mix contained 4 µl of 10X buffer (Fermentas, 0.5 µl $Mgcl_2$, 3 µl dNTPs (Fermentas), and 0.2 µl Taq polymerase (Fermentas). The PCR vial was placed in PCR machine-thermocycler (PRIME, UK). The PCR mixtures were poured in microcentrifuge tubes and vortexed for proper mixing before loading them into the thermocycles. PCR was performed at different cycle's conditions according to the primers as presented in Table 2.

2.5 Statistical Analysis

Statistical analysis was carried out using the SPSS 17.0 statistical software. Chi-square analysis was used to establish the significant relationship in the prevalence of the organism. P value of < 0.05 was considered significant for all the comparisons.

Table 1. Primers used for the PCR amplification of ESBL genes in *Klebsiella* isolates

Primer Name	Sequence (5'-3')	Target Gene	Size (bp)
*bla*SHV-F	AGGATTGACTGCCTTTTTG	SHV	393
*bla*SHV-R	ATTTGCTGATTTCGCTCG		
*bla*TEM-F	CCCCGAAGAACGTTTTC	TEM	517
*bla*TEM-R	ATCAGCAATAAACCAGC		
*bla*CTX-M-F	CGATGTGCAGTACCAGTAA	CTX-M	585
*bla*CTX-M-R	TTAGTGACCAGAATAAGCGG		
AAC-3-IV-F	AGTTGACCCAGGGCTGTCGC	AAC-3-IV	286
AAC-3-IV-R	GTG TGC TGC TGG TCC ACAGC		
*bla*OXA-F	ATATCTCTACTGTTGCATCTCC	OXA	620
*bla*OXA-R	AAACCCTTCAAACCATCC		

Table 2. PCR conditions used for PCR amplification in *Klebsiella* isolates

Target Gene	PCR conditions	
SHV	Initial denaturation for 5min at 94ºC	1 cycle
	94ºC for 30s	35 cycles
	68ºC for 30s	
	72ºC for 60s	
	Final extension at 72ºC for 7min	1 cycle
TEM	Initial denaturation for 5min at 94ºC	1 cycle
	94ºC for 60s	35 cycles
	55ºC for 30s	
	72ºC for 60s	
	Final extension at 72ºC for 10min	1 cycle
CTX-M	Initial denaturation for 5min at 94ºC	1 cycle
	94ºC for 30s	30 cycles
	60ºC for 30s	
	72ºC for 60s	
	Final extension at 72ºC for 7min	1 cycle
AAC-3-IV/OXA	Initial denaturation for 10min at 94ºC	1 cycle
	94ºC for 60s	30 cycles
	55ºC for 60s	
	72ºC for 2mins	
	Final extension at 72ºC for 5min	1 cycle

3. RESULTS

Fig. 1 showed the distribution of *Klebsiella* species of which *Klebsiella pneumoniae* (46%), *Klebsiella oxytoca* (19%), *K. planticola* (10.9), *K. ozaenae* (10.9%), *K. rhinoscleromatis* (9%), and *K. ornithinolytica* (6%). Extended spectrum beta-lactamase detection by Double disk synergy test on Mueller- Hinton agar (Fig. 2). The antibiotic susceptibility profile of the isolates are represented in Table 3. The multiple antibiotic resistance (MAR) pattern of the isolates are depicted in Table 4. The MAR pattern shows twenty-one different pattern to different classes of antibiotics.

Eighty percent (80%) of the *Klebsiella* species were multiple antibiotic resistance showing resistance to three or more different classes of antibiotics. Sixty-one percent of *Klebsiella* isolates (61%) produced extended spectrum beta-lactamase. All ESBLs producing isolates were multi drug resistant and showed resistance to penicillin (100%), beta-lactam (54%), cephalosporins (30-45%) classes of antibiotics. There was a significant difference in the susceptibility pattern of each antibiotics used ($P<0.05$). blaTEM (31%) was the most prevalent followed by AAC (27%). The rates of CTX-M and SHV genes are 21% and 13% respectively, and OXA (8%) (Fig. 3). Fig. 4 showed the agarose gel electrophoresis of the amplification product coding *TEM* gene at 517bp.

4. DISCUSSION

The continual spread and occurrence of ESBL resistance in hospitals in Nigeria is known only to few especially to third generation cephalosporins and beta lactams. However, the rise in treatment failures of this drugs in developing countries has continue to make life difficult for patients, their loved ones and the practicing health care workers. Also in addition to this, inappropriate and personal prescriptions of antibiotics (use and mis-use), lack of proper and sustainable infection prevention and control (IPC) measures in place has made the situation worse. So therefore it was observed in this study that many of the isolates were resistant to augmentin, beta-lactam, penicillin and highly susceptible to ofloxacin, ciprofloxacin and imipenem which are not common in use to the patients. The isolates were resistance to ceftazidime (43%), Cefuroxime (30%), piperacillin (100%) and cefotaxime (37%). The resistance in penicillin was very high compare to that of ceftazidime, cefuroxime and cefotaxime. The prevalence of ESBL-producing *Klebsiella* species in this study which was 61% is lower compared with studies in other countries where the prevalence of ESBL-producing *K. pneumoniae* was 71%, 66.7% in India [14] and Iran (72.1%) [15]. It is much higher than reports from Saudi Arabia (25.2%), United Arab Emirates (41%) [16] and Kuwait (31.7%) [17].

Studies reported that a great percentage of *K. pneumoniae* are ESBL-producers as Omar et al. [18] who reported that the prevalence of ESBL-producing isolates among Enterobacteriaceae members was 25.8% with the highest frequency found in *K. pneumoniae* and Marra et al. [19] stated that 52% of isolates were ESBL-producing *K. pneumoniae* while El-Gendy [20] reported that

Table 3. Antibiotic susceptibility profile of *Klebsiella* species isolates

Antibiotics (ug)	Susceptible (%)	Intermediate (%)	Resistant (%)
Augmentin (AUG)	38.6	7.2	54
Ceftazidime (CAZ)	55.4	1.8	42.7
Ciprofloxacin (CPR)	93.9	1.8	4.2
Cefuroxime (CRX)	65.6	4.2	30.1
Cefixime (CXM)	50	5.4	44.5
Beta-lactam inhibitor (AMC)	27.7	3.6	68.6
Chloramphenicol (CHP)	87.3	0	12.6
Gentamicin (GEN)	84.9	2.4	12.6
Nitrofurantoin (NIT)	93.3	0.6	6.0
Ofloxacin (OFL)	93.3	2.4	4.2
Streptomycin (STP)	8.4	4.8	86.7
Trimethoprim-Sulfamethoxazole (SXT)	72.2	6.0	18.0
Cefotaxime (CTX)	59.0	3.6	37.3
Piperacillin (PRL)	0	0	100
Imipenem (IMP)	95.7	0	4.2
Cefoxitin (FOX)	84.9	0.6	14.4

Table 4. Multiple Antibiotic Resistance (MAR) pattern of *Klebsiella* Species from sputum samples

Bacterial isolates	No of antibiotics	MAR pattern	Number of MAR pattern	Frequency	Overall (%)
Klebsiella **spp**	9	AUG, CAZ, CPR, CTX, CXM, GEN, NIT, OFL, PRL		1	
	8	AUG, CAZ, CPR, CRX, CXM, GEN, OFL, PRL		6	
	7	AUG, CAZ, CRX, CTX, CXM, GEN, PRL		2	
		AUG, CRX, CTX, CXM, FOX, NIT, PRL		2	
		AUG, CRX, CXM, FOX, IPM, NIT, PRL		4	
		CAZ, CPR, CTX, CXM, GEN, OFL, PRL		1	
	6	AUG, CRX, CTX, IPM, NIT, PRL	21	2	
		AUG, CRX, CTX, CXM, GEN, PRL		9	
		AUG, CAZ, CRX, CTX, CXM, PRL		5	
		AUG, CRX, CTX, FOX, OFL, PRL		4	
	5	AMC, CAZ, CRX, FOX, PRL		9	
		AUG, CTX, FOX, IPM, PRL		11	
		AMC, CAZ, CTX, CXM, PRL		11	
		AMC, AUG, CXM, GEN, PRL		4	
	4	AMC, AUG, CAZ, PRL		29	
		AMC, CAZ, GEN, PRL		6	
		AMC, AUG, FOX, PRL		1	
		CAZ, CPR, CXM, PRL		2	
		CAZ, CXM, NIT, PRL		1	
	3	AMC, CAZ, PRL		4	
		AMC, AUG, PRL		19	
		Overall Total		**133**	**80**

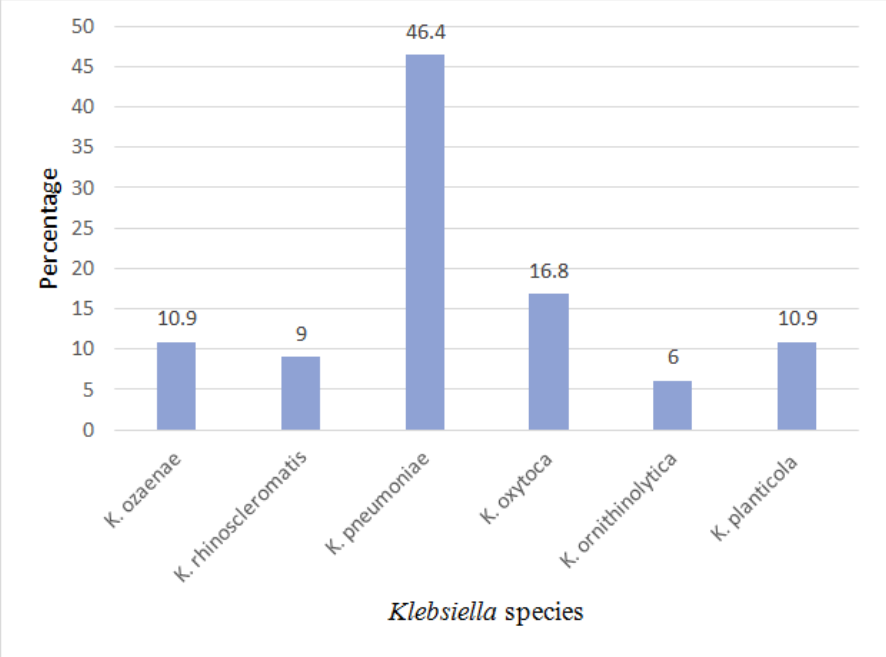

Fig. 1. *Klebsiella* species isolated from sputum and throat swab samples

ESBL-producing *K. pneumoniae* were 51%. In a study carried out in India Institute of Medical Sciences in the year 2001, 68% of isolates were ESBL-producing *Klebsiella* [21]. In Cairo University Hospitals in Egypt, Balbaa et al. [22] showed that 47.8% of *K. pneumoniae* isolates were ESBL-producers.

The overall prevalence of ESBL-producers was found to vary greatly in different geographical areas. This may be the result of differences in the type and amount of antibiotics consumed and differences in the time of collection of isolates. The present study reflects an increase in the prevalence of ESBL-producing *Klebsiella* species (61%) in Nigeria since it was 51% in 2012 [23]. It is of concern in this study that proliferation of beta-lactamase resistance among strains may have been due to misuse of antibiotics, proliferation of resistant clones, transfer of resistance-carrying plasmids and inability to detect emerging phenotypes in developing countries. But most probably in the nearest future, if this irrational use is not stopped, infection with these Gram-negative bacteria increase the rate of resistant to drugs that are now sensitive, resulting in increase in morbidity and mortality.

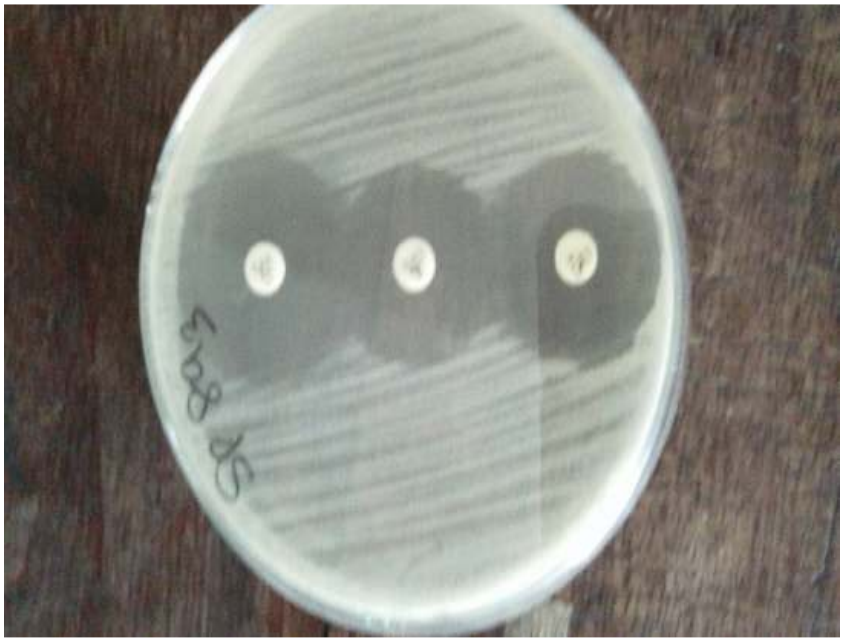

Fig. 2. Plate showing ESBL detection by Double disk synergy on Mueller- Hinton agar

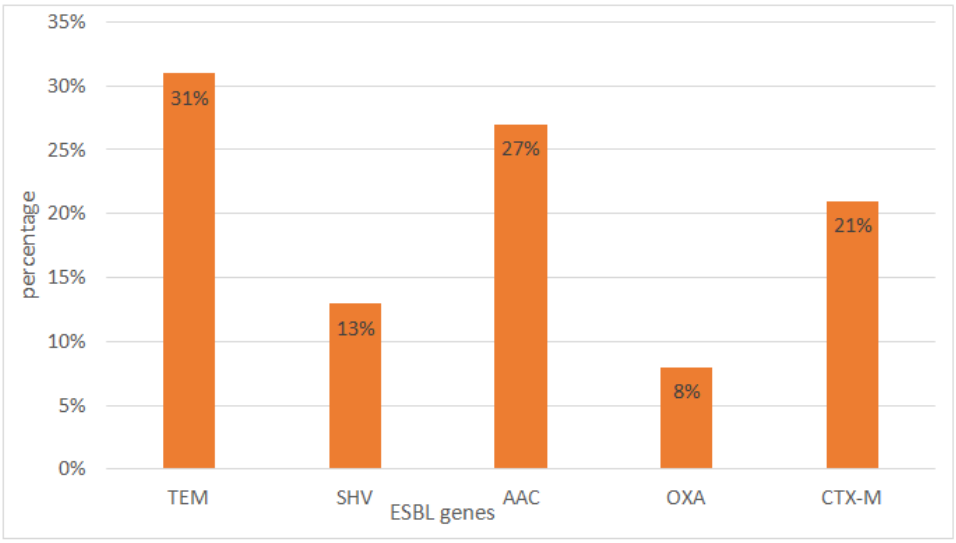

Fig. 3. Distribution of extended spectrum beta-lactamases resistance genes in *Klebsiella* isolates

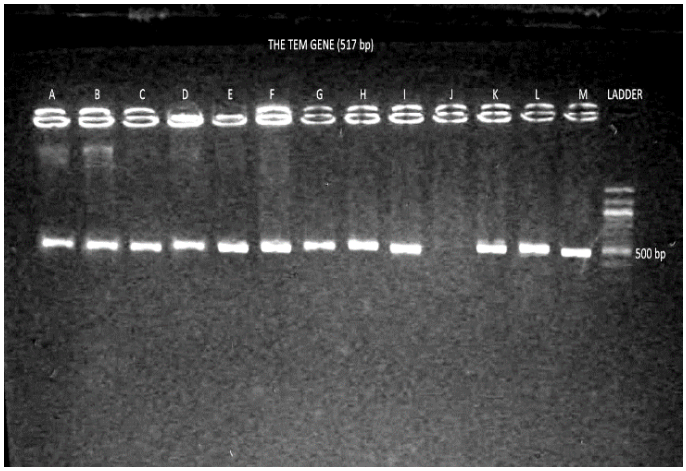

Fig. 4. PCR amplification of TEM gene among ESBL positive Klebsiella species
Ladder=DNA Ladder (100 bp), Lane A-I, K-M = Isolates Positive for TEM gene, Lane J= Negative

Infection-control practitioners and clinicians need the clinical laboratory to rapidly identify and characterize different types of resistant bacteria, especially ESBLs in order to minimise the spread of these bacteria and help to select more appropriate antibiotics.

The most common ESBL type found in this study was TEM (31%), which is the most prevalent. This is similar to the study of Olowe et al. [23] who reported 32% as the prevalent rate of TEM which is higher than the result (23%) obtained in Iran in 2009. This percentage was considered to be a bit higher when compared with 27.3% in *K. pneumoniae* reported by Akram et al. [24] who conducted a survey on the urinary tract isolates in India. The rates of AAC and CTX-M genes are 27% and 21% respectively which agrees with the work done in Iran in 2010 with 24.5% CTX-M prevalence [25].

5. CONCLUSION

This study revealed high antimicrobial resistance among *Klebsiella* isolates in the study region. The ESBL types TEM, SHV, CTX-M, AAC and OXA played an important role in this antimicrobial resistance and the emergence of the ESBLs is increasing in Nigeria. The prevalence of the isolates possessing this resistance enzymes concomitantly has caused serious problems for treatment and diagnosis. Consequently, regular epidemiological assessments on the drug resistance patterns of the isolates and determination of the molecular resistance mechanisms can be useful for the empirical treatment of the infections and appropriate preventive policy in hospitals to control further spreading of these resistant strains.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Shahid M, Sobia F, Singh A, Khan Haris M, Hawkey PM, Huq A. AmpC ß-lactamases and bacterial resistance: An updated mini review. Rev. Med. Microbiol. 2009;20(3):41–55.
2. Sundin DR. Hidden beta-lactamases in the enterobacteriaceae – Dropping the extra disks for detection, Part II. Clin Microbiol, Newsletter. 2009;31(7):47–52.
3. Leylabadlo HE, Kafil HS, Yousefi M, Aghazadeh M, Asgharzadeh M. Persistent infection with metallo-beta-lactamase and extended spectrum β-lactamase producer Morganella morganii in a patient with urinary tract infection after kidney transplantation. J. Nat. Sci. Biol. Med. 2016;7(2):179.5.
4. Oli AN, Eze DE, Gugu TH, Ezeobi I, Maduagwu UN, Ihekwereme CP. Multi-antibiotic resistant extended-spectrum beta-lactamase producing bacteria pose a challenge to the effective treatment of wound and skin infections. Pan Afr. Med. J. 2017;27:66.

5. Maina D, Makau P, Nyerere A, Revathi G. Antimicrobial resistance patterns in extended-spectrum β-lactamase producing Escherichia coli and Klebsiella pneumoniae isolates in a private tertiary hospital, Kenya. Microbiol. Discov. 2013;1(5):1-4.
6. Oluduro AO, Aregbesola OA, Fashina CD. Extended Spectrum Beta- Lactamase-Producing Uropathogenic Escherichia coli in Pregnant Women Diagnosed With Urinary Tract Infections in South-Western Nigeria. J. Mol. Biol. Res. 2014;4(1):34-41.
7. Kalantar D, Mansouri S. Emergence of multiple ß-lactamases produced by Escherichia coli clinical isolates from hospitalized patient in Kerman, Iran. Jundishapur J Microbiol. 2011;3(4):137.
8. Paterson DL. Resistance in Gram-negative bacteria: Enterobacteriaceae. Am J Med. 2006;119(6 Suppl 1):S20–8.
9. Vasaikar S, Obi L, Morobe I, Bisi-Johnson, M. Molecular Characteristics and Antibiotics Resistance Profiles of Klebsiella isolates in Mthatha, Eastern Cape Province, South Africa. Int. J. Microbiol. 2017; Article ID 8486742,7pages

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10. Perez F, Endimiani A, Hujer KM, Bonomo RA. The continuing challenge of ESBLs. Curr. Opin. Pharmacol. 2007;7(5):459–69.
11. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in European Euro Surveillance. 2008;13(47).
12. Bush K, Jacoby GA. Updated functional classification of beta lactamases. Antimicrob. Agents Chemother. 2010; 54(3):969–76.
13. Cockerill FR. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement.: Clinical and Laboratory Standards Institute (CLSI); 2011.
14. Hoda Hassan BA. Molecular characterization of extended-spectrum beta-lactamase producing Entero-bacteriaceae in a Saudi Arabian tertiary hospital. 2014;13;8(3):282-8.
15. Feizabadi MM, Raji N, Majnooni A, Aligholi M, Shahcheraghi F, Parvin M, Yadegarinia D. Distribution of bla(TEM), bla(SHV), bla(CTX-M) genes among clinical isolates of Klebsiella pneumoniae at Labbafinejad Hospital, Tehran, Iran. Iran Microb. Drug Resist. 2010;16:49-53.
16. Al-Zarouni MSA, Rashid F, Al-Jesmi SM, Panigrahi D. Prevalence and antimicrobial susceptibility pattern of extended-spectrum beta-lactamase-producing Enterobac-teriaceae in the United Arab Emirates. Med. Princ. Pract. 2008;17:32-6.
17. Mokaddas EM, Shati S, Rotimi VO. The technical aspects and clinical significance of detecting extended-spectrum beta-lactamase-producing Enterobacteriaceae at a tertiary-care hospital in Kuwait. J. Chemother. 2008;20:445-51.
18. Omar M, Badawi H. Helmi H. Comparison of different methods for detection of ESBLs and their genetic relatedness among Enterobacreiaceae clinical isolates in a research medical institute. Egypt J. Med. Microbiol. 2001;10(2):365-75.
19. Marra AR, Pereira CAP, Castelo A, De Carmo Filho JR, Cal RGR, Sader HS, Wey SB. Health and economic outcomes of the detection of Klebsiella pneumoniae-produced extended-spectrum b-lactamase (ESBL) in a hospital with high prevalence of this infection. Int. J. Infect. Dis. 2006;10: 56-60.
20. El-Gendy SG. Characterization of extended-spectrum b-lactamase producing Klebsiella species from nosocomial infected cases in ICUs in Assiut University Hospitals. Thesis for the Master Degree in Medical Microbiology and Immunology, Assiut University, Egypt; 2006.
21. Mathur P, Kapil A, Das B, Dhawan B. Prevalence of extended spectrum beta lactamase producing gram negative bacteria in a tertiary care hospital. Indian J. Med. Res. 2002;115:153-7.
22. Balbaa A, Gohar H, Ezzat H, Abouzeid M, Hussein AM, Eissa S. Study of microbiological pattern of hospital and community acquired infection isolates: Minimal inhibitory concentration, beta-lactamase production and plasmid profile analysis. Egypt. J. Med. Microbiol. 1999;8:333-342.
23. Olowe OA, Oladipo GO, Makanjuola, OA, Olaitan JO. Prevalence of Extended Spectrum Beta-Lactamases (ESBLS) carrying genes In Klebsiella spp from clinical samples at Ile-Ife, South Western Nigeria. Int. J. Pharm. Med. Biol. Sci. 2012;1(2):129-138.

24. Akram M, Shahid M, Khan AU. Etiology and Antibiotic Resistance Patterns of Community-Acquired Urinary Tract Infections in JNMC Hospital Aligarh, India. Ann. Clin. Microbiol. Antimicrob. 2007;6(4).

25. Nasehi L, Shahcheraghi F, Nikbin VS, Nematzadeh S. PER, CTX-M, TEM and SHV Beta-Lactamases in Clinical Isolates of Klebsiella pneumoniae Isolated from Tehran, Iran, Iran J. Basic Med. Sci. 2010;13(3):111-118.

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