

Original Research Article

Presence of bla TEM and bla SHV Genes in ESBLs Producing *Klebsiella Pneumonia* among Female Patients in University of Benin Teaching Hospital

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ABSTRACT

This project was carried out to determine the presence of *bla* TEM and *bla* SHV genes in ESBL producing *Klebsiella pneumonia* from urogenital tract of female patients at UBTH. The specific objectives of the study were: (i) to isolate the multi drug resistant strains of *Klebsiella pneumonia* in urogenital tract of female patient at UBTH, (ii) to identify and characterize ESBLs in the multi-drug resistant *Klebsiella pneumonia* strains, (iii) to determine what fraction of potential ESBL producers go on to be confirmed, (iv) to determine what fraction of the isolates are hospital or community acquired. Isolates were taken from women who had urogenital infection from both the inward and outward patients, a total of 200 (two hundred) *klebsiella pneumoniae* isolates which was broken into 100 (one hundred) inward patients and 100 (one hundred) outward. Table 2.0 shows the biochemical identification of the isolates, *Klebsiella pneumoniae* is a lactose fermenter, it is a motile organism, citrate and urease positive, utilizes glucose to produce acid and gas, indole negative and Hydrogen sulphide negative. The emergence and provocative prevalence and its continuous spread of ESBL-producing *Klebsiella pneumonia* strains in this study are worrisome which brings to the ineffective usage of Cephalosporins against these isolates. More new antibiotics are drum rolled to inhibit the production of these enzymes such agents like clavulanic acid has been over powered by their over production of the enzymes bringing challenges to pharmacotherapeutics. Although imipenem is the drug of choice for serious infection disease these days, prolong and extensive use of this drug in treatment of infection caused by resistant isolates would be enhanced as there is newer evidence of imipenem resistance.

Keywords: ESBLs, blaTEM, BlaSHV, Beta-lactamases.

INTRODUCTION

Extended spectrum of beta-lactamases (ESBLs) are enzymes that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, carbapenems and the monobactam aztreonam. They're a global public health trouble as infections caused by such enzyme producing organisms could lead to higher morbidity and mortality rate. [1] Cephalosporins and other beta-lactams have ability to destroy bacteria by inhibiting

essential steps in the bacterial cell wall synthesis which in the end, results in lysis and death of the bacterial cell. [1] These antibiotics all have a common element in their molecular structure: a four-atom ring known as a beta-lactam. The beta lactamase confer antibiotic resistance by breaking the antibiotic structure. This it does by breaking the beta-lactam ring open through hydrolysis deactivating the molecular antibacterial properties. ESBLs are often plasmid mediated and most of the enzymes

are members of TEM and SHV families [2,3] that have been described in many countries. The TEM was first reported in *E.coli* isolated from a patient named Temoniera in. [4] The name of the other beta-lactamase, SHV, is due to sulf-hydryl variable active site. [5] Almost 160 variants of TEM-1 and TEM-2 penicillase have been described, many of which exhibit activity against extended-spectrum cephalosporins. Class A enzymes are mainly plasmid encoded, and the first to be described at amino acid sequence level were the enzymes TEM-1 and TEM-2. [4] The SHV family has been derived from *Klebsiella* spp. SHV-1 is universally found in *K. pneumonia*, [5] evolved as a chromosomal gene in *Klebsiella* spp., and was later incorporated into a plasmid, which has spread to other enterobacteria species. [6] A total of 40 types of SHV have already been reported. Extended spectrum beta-lactamases are often plasmid mediated derived from mutations in the classic TEM and SHV genes by one or more amino acid substitution around the active site. Detection of bla TEM and bla SHV genes by molecular methods in ESBL producing bacteria and their occurrence can provide useful information about its epidemiology and risk factors associated with these infections. This project was carried out to determine the presence of bla TEM and bla SHV genes in ESBL producing *Klebsiella pneumonia* from urogenital tract of female patients at UBTH. The specific objectives of the study were: (i) to isolate the multi drug resistant strains of *Klebsiella pneumonia* in urogenital tract of female patient at UBTH, (ii) to identify and characterize ESBLs in the multi-drug resistant *Klebsiella pneumonia* strains.(iii) to determine what fraction of potential ESBL producers go on to be confirmed.(iv) to determine what fraction of the isolates are hospital or community acquired.

MATERIALS AND METHODS

Samples were collected from University of Benin Teaching Hospital

(UBTH), UBTH is a Federal tertiary hospital founded in 1973. It's located in Benin City, Edo State, Nigeria. Isolates of *Klebsiella pneumonia* from females with urogenital tract infection from both the In-patient and the out-patients unit in the University of Benin Teaching Hospital Microbiology department were collected. A total of 200 isolates of *Klebsiella pneumonia* from females with urogenital tract infection were collected for this study, 100 isolates where from the in- patient and 100 from the out-patient all from the University of Benin Teaching Hospital, Benin City, Edo State. Materials used include bijou bottles, petri dishes, Muller Hinton agar, Nutrient agar, MacConkey agar, peptone water, hot air oven, incubator, burner and gas, hand gloves, chemical balance, distilled water, refrigerator, Kovac's reagent, oxidase reagent (hydrogen peroxide), filter paper, wire loops, gram stain reagents, microscopic slides, autoclave, cotton wool, antibiotic disc. All prepared agar and liquid medium, antibiotic disc, Kovac's reagent were stored at 2⁰C to 8⁰C and were used within the specified period of viability. Other materials were kept at room temperature. Isolates were collected from already cultured plate in University of Benin Teaching Hospital Microbiology Department. Working under aseptic conditions isolates collected were sub-cultured onto MacConkey agar plates, and incubated aerobically at 37⁰C for 24hours to obtain pure colonies. After 24 hours of incubation, the sub-cultured samples were confirmed. The identity of the isolates was based on the colonial morphology, motility, gram stain, sugar fermentation and utilization of standard biochemical tests. [7,8] The screening of isolates for ESBL production was carried out firstly by determining their resistance to any of the third generation Cephalosporins. AST was done by standard disc-agar diffusion technique on Muller Hinton agar (Oxoid, UK). The overnight pure culture of the isolates were suspended in sterile water and the turbidity matched with 0.5% of

McFarland standard, once matched, this was used to flood the surface of the Muller-Hinton agar. Excess was drained off and the plate allowed to dry. Appropriate antibiotics disc of different antimicrobial action were aseptically placed on the inoculated plate and pressed firmly to ensure complete contact with the agar. Single antimicrobial disc of Cefotaxime, Ceftazidime, Ceftriaxone, Augmentin, Aztreonam, were used in this study. The plates were further incubated for 24 hours at 37°C. After 24 hours the diameter of the zone, surrounding each antimicrobial agent was measured in millimeter (mm) using a ruler and the results were interpreted as Susceptible (S) or Resistant (R) according to the CLSI (2010). The double disc synergy test was performed on Muller Hinton agar on isolates showing resistance to any of the third generation cephalosporins used for the screening. An Amoxicillin/Clavulanic disc was placed at the center of the inoculated plates and the disc containing the standard 30µg of Ceftazidime, Ceftriaxone, Cefotaxime, aztreonam were placed 20mm apart (Centre to Centre) from the Augmentin (20µg amoxicillin and 10ug clavulanic acid) disc. Disc placement was expedited by melting holes in the lid of the petri dish and equally using the lid as a template to mark the

bottom of the agar plate for proper and accurate disc location. Inoculated media were incubated overnight at 37°C. An enhanced zone of inhibition and synergy between any of the beta lactam disc and the amoxicillin-clavulanic disc was interpreted as a presumptive evidence for the phenotypic presence of extended spectrum beta lactamase. Isolates with this pattern were indicative and recorded as positive for ESBL production. The isolates positive for ESBL production were taken to International Institute of Tropical Agriculture, Molecular Laboratories, Ibadan. ESBL positive isolates were cultured in LB (Luria-Berneti) broth at 37 °C overnight and plasmid DNA was extracted according to the method of Johnson and Woodford (1998). Specific primers and annealing temperature for amplifying the blaSHV and blaTEM, genes by PCR were shown in Table 1. PCR was carried out in solution containing 200 µM concentration of dNTPs, 10 Pmol of each primer, 0.8 mM/µl MgCl₂, 0.5 U *Taq* polymerase and 50 ng DNA template in a final volume of 25 µl *Klebsiella pneumoniae* 6681 containing blaSHV and blaTEM gene (Kindly provided by Patrice Nordmann) were used as controls.

Table 1.0: Distribution across the primer, the names, annealing temperature and the genes

Primer Name	SEQUENCE (5' TO 3')	SIZE (BP)	ANNEALING TEMPERATURE	GENE	REFERENCE
TEM A	GAGTATTCAACATTTCCTGTGTC	848	43	bla TEM	36
TEM B	TAATCAGTGAGGCACCTATCTC	846	43	bla TEM	36
SHV A	AAGATCCACTATCGCCAGCAG	231	60	blaSHV	35
SHV B	ATTCAGTTCGGTTCCAGCGG	231	60	blaSHV	36

Key: TEM=Temonera, SHV=Sulphydryl-variable

The SHV and TEM ESBL amplified genes were characterized by PCR-Restriction Fragment Length Polymorphism (RFLP). This analysis was performed using *Pst*I for TEM and PER and *Pvu*II for SHV ESBL amplified genes. Restriction fragments were analyzed using gel electrophoresis in a 1% (W/V) agarose gel.

STATISTICAL ANALYSIS

Analysis by percentage was used

RESULTS

Isolates were taken from women who had urogenital infection from both the inward and outward patients, a total of 200 (two hundred) *klebsiella pneumoniae* isolates which was broken into 100 (one hundred) inward patients and 100 (one hundred) outward. Table 2.0 shows the biochemical identification of the isolates, *Klebsiella pneumoniae* is a lactose fermenter, it is a motile organism, citrate and urease positive, utilizes glucose to

produce acid and gas, indole negative and Hydrogen sulphide negative.

Table 2.0. Biochemical table of isolate identifying *Klebsiella pneumonia*

Isolate	Motility	Indole	Oxidase	Urease	Citrate	H2S	Glucose	Methyl-red	Lactose
<i>Klebsiellapnuemoniae</i>	+	-	-	+	+	-	A/G	-	+

Key: += Positive, --=Negative, A=Acid G=Gas

The total number of *Klebsiella pneumoniae* from out-patient was 100. The susceptibility pattern resistant are as follows: tetracycline (100%), imipenem (40%), ceftazidime (70%), augmentin (72%), cefotaxime (76%), ampicillin (77%), ceftriaxone (90%) respectively.

The total number of *Klebsiella pneumoniae* isolates from inpatient was 100. The susceptibility pattern resistant are as follows: tetracycline (80%), imipenem (20%), ceftazidime (61%), augmentin (60%), cefotaxime (50%), ampicillin (63%), ceftriaxone (80%) respectively. All these are shown in table 3.0 below

TABLE 3.0 Susceptibility profile across the clinical isolate

ISOLATE	CN	AMP	AUG	CTX	CAZ	CRO	IM	TET	OFL
Outpatient (n=100)									
<i>Klebsiella p</i>									
R									
S	65	77	72	76	70	90	40	100	60
	35	23	28	24	30	10	60	---	40
In Patient (n=100)									
<i>Klebsiella p</i>									
R									
S	45	63	60	50	61	80	20	78	43
	55	37	40	50	39	20	80	22	57

Key: CN=Gentamicin, AMP=Ampicillin, AUG=Augmentin, CTX=Cefotaxime, CRO=Ceftriaxone, IM=Imipenem, TET=Tetracycline, OFL=Ofloxacin, R=Resistant, S=Susceptible, n=total number of isolates

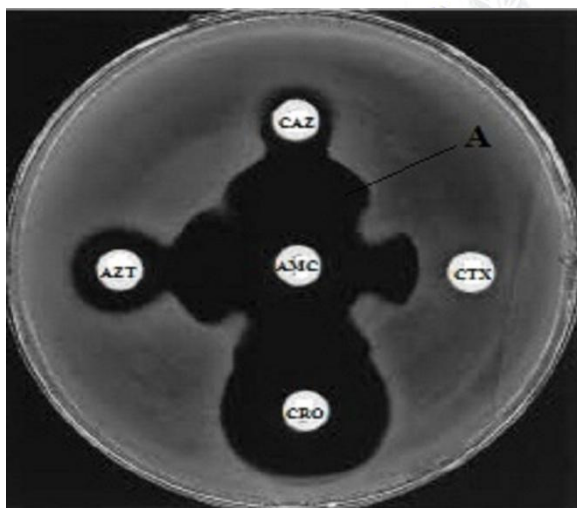


Figure 1.0 Double disk synergy test, for the phenotypic presumptive detection of Extended spectrum beta lactamase positive ESBL

DOUBLE DISC SYNERGY TEST (DDST)

The total number of *Klebsiella pneumoniae* isolates collected for this study was phenotypically screened for ESBL production. A total of 15% and 25% from the out-patient and in-patient respectively

were ESBL producers as shown in figure 1.0 below. The overall positive phenotypic ESBL producers from both the In-patient and Outward patient was at 20% in total percentage

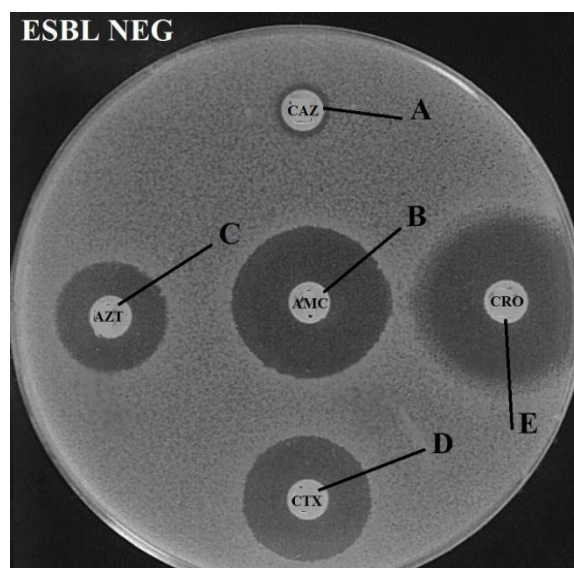


Figure 2.0 Double disk synergy test, for the phenotypic presumptive detection of Extended spectrum beta lactamase Negative ESBL

Table 4.0 Distribution of ESBL producer across the various isolate Phenotypic presumptive confirmation using a double disk synergy test DDST

DEMOGRAPHY Isolate (<i>K. pneumonia</i>)	P	N
IN PATIENT n=100	25 (25%)	75(75%)
OUT PATIENT 100	15(15%)	85(85%)
Total	40(20%)	160(80%)

Key: P=Positive, N=Negative, n=total number of isolates

4 PCR ANALYSIS

The 15% out-patient and 25% in-patient isolates that were ESBL positive were taken to IITA and their DNA was extracted, amplified, sequenced and characterized. Out of the 15% of out-patient, beta lactamase (*bla*) TEM was present in 7 (46%) isolates, 4 (26%) carried the *bla* SHV.

From the 25% of in-patient, *bla* TEM was present in 8 (32%) isolates and 3 (12%) carried the *bla* SHV as shown in table 5.0 and figure 3.0a and b below

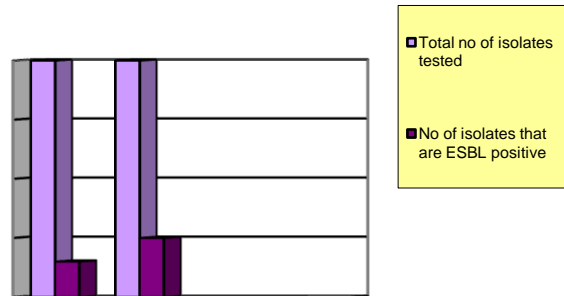


Table 5

<i>K. pneumonia</i> Isolates	TOTAL NUMBER OF ESBL POSITIVE	Bla TEM	Bla SHV	Bla TEM/Bla SHV
IN PATIENTS	25	8 (32%)	3 (12%)	2(8%)
OUT PATIENTS	15	6(40%)	5 (33%)	2(13%)

Key: TEM=Temonera, SHV=Sulphurhydryl Variable

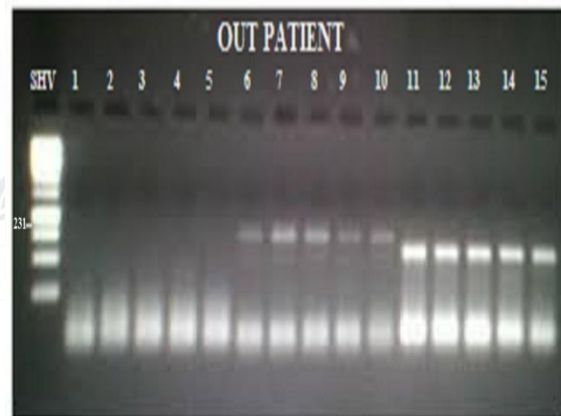
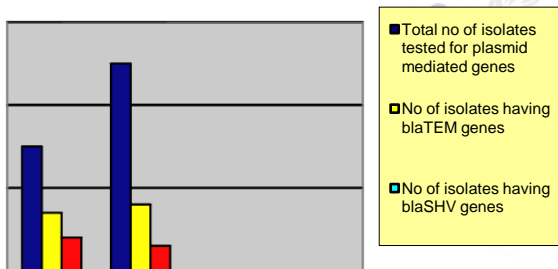


Fig 3.0b SHOWS THE PCR GENOTYPIC PRESENCE OF BLA SHV GENES ACROSS THE OUT PATIENT

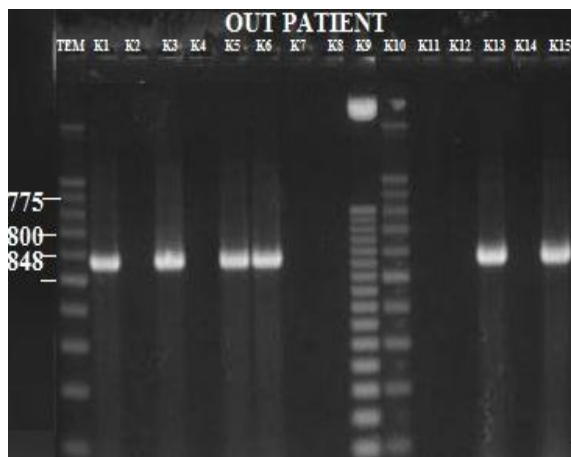


Fig 3.0a SHOWS THE PCR GENOTYPIC PRESENCE OF BLA TEM GENES ACROSS THE OUT PATIENT

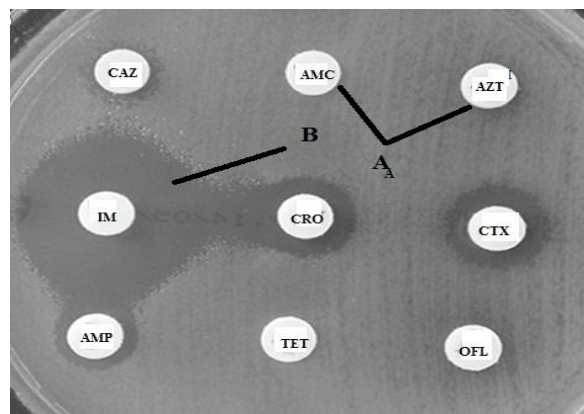


Figure 4.0 Susceptibility profile of the clinical isolate (A) Resistant to Augmentin and Aztreonam, ofloxacin, ampicillin, tetracycline, ceftazidime- (B) Zone of inhibition susceptible Imipenem

Table 5.0 above shows the presence of bla TEM and SHV from the isolates that where ESBL positive phenotypically, a total of 8 and 3 isolate had the presence of the plasmid mediated resistance In the In-patient. From the Out-patient 7 and 4 isolates had the plasmid mediated resistance.

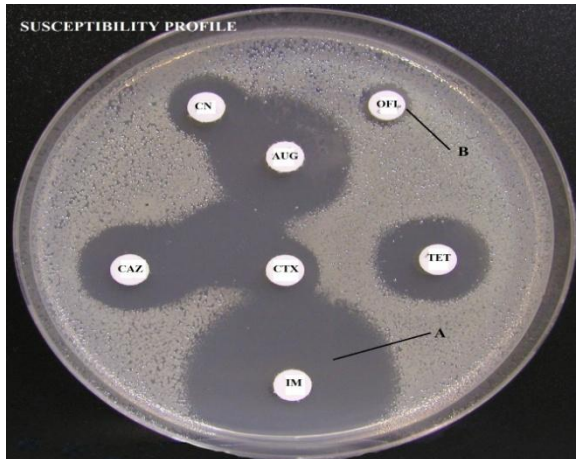


Figure 5.0 susceptibility profile (A) zone of inhibition susceptible (B) Resistant no zone of inhibition

DISCUSSION

All isolates were gotten from women who had presented themselves with urogenital tract infection. Standard morphological and biochemical tests were carried out to properly identify this pathogenic isolate. This was in tune according to [9]. *Klebsiella pneumoniae* is the third most prominent urinary tract pathogen to colonise the female urinary tract and cause infectious process. This also is in tandem with past works, [9,10] most women who have a urogenital infection are usually symptomatic in nature while some others are carriers (asymptomatic) although in our study women with symptomatic presentation of urogenital infection were further taken to the laboratory for microbiological analysis. The susceptibility profile of *Klebsiella pneumoniae* from the female urogenital tract showed a remarkable multi/extended drug resistance with almost 70% of the isolate resistant to one or more of the antibiotics challenged against the isolate, they ranged from the aminoglycosides, the beta-lactam producers and their sulphurhydryl variable which is in

tandem with recent study on drug resistance amongst the *Klebsiella species* isolated from the female urogenital tract [11] also in another study it was observed that ESBL positive isolates exhibited high levels of multi drug resistance. There was an increased resistance of *Klebsiella pneumoniae* to Imipenem which seems to be on the increase with 40% and 20% (fourty and twenty percent) resistant from both the out-patient and in-patient. This can either be as a result of previous drug abuse and less of nosocomial infections as seen in our study that shows more of the drug resistance to Imipenem came from the out-patient unit, arguably some studies believe that nosocomial infection is the leading cause of drug resistance as against non-patient compliance or antibiotic abuse due to over-the-counter administration. The double disc synergy test as a producer of beta lactamase (an enzymatic mode of drug resistance), showed 25% ESBL producer for in-patient and 15% ESBL producer for out-patient, some other topic would argue in the same direction as a previous work done shows a 23% positivity rate. A study from Zaria recovered *Klebsiella pneumoniae* from patient suspected to have urinary tract infections 35.3% were ESBL producers, [12] Presence of ESBL producing *Klebsiella* around the world varies between 3% -8% to 100%. [13,14] The In-patient unit showed a remarkable 25% positivity while the Out-patient showed a 15% which brings us back to our argument of prolong hospitalization and nosocomial infection. These results from our study is in tandem with past issues and further goes a long way to confirm the prevalence of the ESBL producing *Klebsiella pneumoniae* against the Aminoglycosides, the cephalosporins and the fluoroquinolones. blaSHV and the blaTEM (Sulphurhydryl variable and Temonera) in this study in-patient showed a 32% blaTEM and a 12% blaSHV, and 8% blaTEM/SHV, the out-patient had 40% blaTEM, 33% blaSHV and 13% blaTEM/SHV. Among the two ESBL genotypes, the most prevalent genotype was

found to be TEM, this agrees with the study done by, [15] this is also in line with past works which have studied the occurrence of this drug resistant gene as seen in occurrence of these drug resistant genes in Benin metropolis with a 17.1% and 17 % respectively and some other topic carried out in Iran [16] with a 67 and 16% occurrence of the *bla*TEM and SHV genes. *Klebsiella* are opportunistic pathogens and can give rise to severe diseases such as septicemia, pneumoniae, UTI, and soft tissue infection. Typically *klebsiella* infection are nosocomial. The hospitalized, immune compromised patient with underlying diseases is the main target of these bacteria. Thus, *klebsiella* infections serve as a paradigm of hospital acquired infections. [17] From this study we had 100 *Klebsiella pneumonia* isolates from out-patients and 100 isolates from in-patients. 25% of the out-patient isolate were ESBL producer while 15% from out-patient, therefore more EBL producers were gotten from in-patient. This could be as a result of the fact that multi drug resistant strains are found in nosocomial infection and *K. pneumonia* is a nosocomial infection. Some studies believe that nosocomial infection is the leading cause of drug resistance as against non-patient compliance or antibiotic abuse due to over-the-counter administration. Patients having infections caused by ESBL producing organism are at increased risk of treatment failure with broad spectrum beta lactam antibiotics, regardless of the susceptibility test result. With the spread of ESBL producing strains in hospitals all over the world, it is necessary to know the prevalence of ESBL positive strains in a hospital so as to formulate a policy of empirical therapy in high risk units where infection due to resistant organism is much higher. [18]

CONCLUSION

The emergence and provocative prevalence and its continuous spread of ESBL-producing *Klebsiella pneumonia* strains in this study are worrisome which

brings to the ineffective usage of Cephalosporins against these isolates. More new antibiotics are drum rolled to inhibit the production of these enzymes such agents like clavulanic acid has been over powered by their over production of the enzymes bringing challenges to pharmacotherapeutics. Although imipenem is the drug of choice for serious infection disease these days, prolong and extensive use of this drug in treatment of infection caused by resistant isolates would be enhanced as there is newer evidence of imipenem resistance. Due to this malaise the prudent use of beta lactam antibiotics containing an oxyamino group and consistent application of basic infection control procedures in treatment centers is necessary to prevent the rise of this drug resistant organism. Rapid identification of ESBL producing isolates should be adopted in clinical laboratories based on the CCLS recommendation for confirming ESBL production in enterobacteraceae species unfortunately there seems to be a gap between the clinical settings and the laboratories in Nigeria. Due to rare co-operation from patient one must take cognizance that the only effective way of controlling this malaise is the cooperation between the patient, clinical staff and the Laboratory. To control the spread of ESBL producing pathogens appropriate infection control intervention should be implemented for all patients who are infected or colonized with ESBL producing bacteria. These interventions include effective hand hygiene and instituting contact precaution for all colonized and infected.

REFERENCES

1. Dhillon, R.H.P., and Clark, J. (2011). ESBL: a clear and present danger? *Critical Care Research and Practice* Volume 2012 (2012), Article ID 625170, 11 pages <http://dx.doi.org/10.1155/2012/625170>
2. Behrouzi, A., Rahbar, M., and Vand, Y. (2010). Frequency of extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumonia* isolated from urine in an Iranian 1000-bed tertiary care hospital. *African Journal of microbiology resistance*. 4:881-884

3. Mohseni, M and Ramzanzandeh, B. (2012) The role of nanotechnology in automotive industries. Doi 105772/49939.
4. Mansouri, M., and Ramazanzadeh, R. (2009). Spread of extended- spectrum beta-lactamase producing Escherichia coli clinical isolate in sanandaj hospitals. Journal of biological science. 9:362-366
5. Sturenburg, E., and Mack, D. (2003). Extended spectrum beta-lactamases: implications for the clinical microbiology laboratory, therapy and infection control. J.infect. volume 47, issue 4, pages 273-295.
6. Livermore, D.M. (1995). Beta-lactamase in laboratory and clinical resistance. Clinical microbiology review. Volume 8, issue 4, pages 557-584.
7. Brooks, G.F., Butel, J.S., and Morse, S.A. (2001). Jawatz, Merick, Adeiberg's. Medical Microbiology 22nd ed. Pages 217-228.
8. Cheesbrough, M. (2010). District Laboratory Practice in Tropical Countries, part 2. Pages 1778-180.
9. IbehNnanna Isaiah., Bikwe, Nche Thomas., IbehGeorginNwagwu., and Ibeh Isaiah Nnanna. (2011). Incidence of temonera, sulphuhydryl variables and cefotaximase genes associated with β -lactamase producing escherichia coli in clinical isolates; *North American Journal of Medical Sciences* 3;12.
10. IbehNnanna Isaiah. (2016). Bacteruria Associated Urogenital Infections Amongst Women "a Study On Assymptomatic And Symptomatic Presentation In Benin Metropolis, Nigeria": *American Journal of Microbiology and Immunology* 1;(1) 0041-0045.
11. Paterson, D.L., Ko, W.C., and Von Gottberg, A. (2004). Implications of extended spectrum beta-lactamase production in nosocomial infections international prospective study of *Klebsiella pneumoniae* bacteremia. *Annals of Internal Medicine*. 140:26-32.
12. Olonitola, O.S., Olayinka, A.T., Inabo, H.I., and Shaibo, A.M. (2007). Short communication: production of extended spectrum beta lactamasees of urinary isolates of Escherichia coli and klebsiellapneumoniaeinahmadubello university teaching
13. Chanwit, T., Somporn, S., and Wararat C. (2007). A correlation between phenotypes and genotypes of extended spectrum beta lactamase (ESBL) producing klebsiellapneumoniae in university hospital, Thailand. *Journal of infectious disease and antimicrobialagent*. 24:117-23
14. Cristina, M.F., William, A.F., and Nayanne, C.O. (2011). Extended-spectrum beta lactamaseproducing bacteria isolated from haematologic patients in manaus, state of amazonas, brazil. *Brizilian Journal of microbiology*. 42:1076-1084.
15. Begum, F., and Famle, A.S. (2015). Prevalence and Characterization of extended spectrum beta-lactamase production in clinical isolate of Klebsiella pneumonia. *Journal of medical microbiology and diagnosis*. 4:178. doi: 10.4172/2161-0703.1000182
16. Feizabadi, M.M., Delfani, S., and Raji, N. (2010). Distribution of bla(TEM), bla(SHV), bla(CTX-M) genes among clinical isolates of *Klebsiellapneumoniae* at Labbafinejad hospital, Tehran, Iran. *Microb Drug Resist*. 16:49-53.
17. Podschum, R., and Ullmann, U. (1998) klebsiella spp. As nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical microbial review* 11:589-603. PMC 88898.
18. Mathur, P., Kapil, A., Das, B., and Dhawan, B. (2002). Prevalence of extended spectrum betalactamase producing gram negative bacteria in a tertiary care hospital. *Indian journal of medical research* 115:153-157

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