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RESEARCH ARTICLE

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF SHV, TEM, OXA AND EXTENDED-SPECTRUM β-LACTAMASE PRODUCED BY *KLEBSIELLA PNEUMONIA* ISOLATES IN A ADJARA HOSPITAL

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ABSTRACT

The results marked the high prevalence of gene TEM.SHV, OXA, The majority of the strains carried two or more genes. This study points out the high resistance ESBL producing by *Klebsiella pneumonia* sample. A total of 28 clinical isolates of extended spectrum β- lactamase producing *Klebsiellapneumonia*e were sampled in Adjara region. The ESBL resistance was screened using disc diffusion method, while the resistance genes were detected by polymerase chain reaction (PCR). All sample were highly resistance to AML Amoxicilina, CTX Cefotaxima, FEP Cefepime, ATM Aztreonamo, CXM Cefuroxime, CAZ Ceftazidima, PRL Piperacilin, AUG Amox. + Ac. Clavulanico, TE Tetraciclin, STX Sulfamet. + Trimetrop-resistanec profile - 100%, F Nitrofurantoina, TOB Tobramicin- 84, 21%, ETP Ertapenemo- 78, 94%, C Cloranfenicol, CIP Ciprofloxacino, LEV Levofloxacin- 78, 94%, PIP/TAZ Piperacilin/Tazobactam, CN Gentamicina-68, 42%, IMI Imipenemo, MRP Meropenemo, AK Amikacin - 57, 89%, NET Netilmicin-53%.

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INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) were first described in the 1980s in Klebsiella species and later they have been detected in E. coli and other genera of the Enterobacteriaceae family. ESBLs are the rapidly evolving group of β-lactamase enzymes whichhave the ability to hydrolyze all cephalosporins and monobactams, but are inhibited by β-lactamase inhibitors, such as clavulanic acid (Bali *et al*., 2010). ESBLs are also able to hydrolyze 3 and 4 generation cephalosporins and monobactams. ESBL producing strains are inhibited by - lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) **(**Bradford, 2001; Pitout, 2007and Giraud-Morin, 2003).

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ESBLs are a group of enzymes encoded by genes described predominantly on plasmid that are common among Enterobacteriaceae (Poole, 2004). ESBL are an increasingly important cause of transferable multidrug resistance in gram-negative bacteria throughout the world. These bacteria have spread rapidly and have become a serious threat to human health worldwide (Giraud-Morin, 2003; Poole, 2004 and Gupta, 2007). ESBL are an important cause of transferable multidrug resistance in gram-negative bacteria throughout the world. These bacteria have spread rapidly and have become a serious threat to human health worldwide. Determination of ESBL genes by molecular techniques in ESBL producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections (Bali *et al*., 2010; Al-Agamy *et al*., 2009).

MATERIALS AND METHODS

Antimicrobial susceptibility testing

Disk diffusion method was performed to test the susceptibility of *Klebsiella pneumonia* isolates to common antibiotics on Mueller-Hinton agar, with an inoculum equal to 0.5 McFarland turbidity according to CLSI [16]. The plates were incubated at 37°C for 18-24 hrs. and the inhibition zone diameters around the antibiotic discs were measured. There were samples of sputum, urine and samples of biological fluids. All isolates were examined for the antibiotic resistance of the following antibiotics: β-LACTÂMICOS-AML Amoxicilina, CTX Cefotaxima, FOX Cefoxitina, FEP Cefepime. MONOBA CTAMO-ATM Aztreonamo, EFT Ceftiofur, CAZ Ceftazidima, CPT Ceftarolina; β-LACTÂMICOS + INIB. β-LACT-AUG Amox. + Ac. Clavulanico, ETP Ertapenemo, TE Tetraciclina, IMI Imipenemo, CT Colistina, MRP Meropenemo, C Cloranfenicol, DOR Doripenemo, TGC Tigeciclina, PIP/TAZ Piperacilin/Tazobactam, CIP Ciprofloxacino, LEV Levofloxacin, STX Sulfamet. + Trimetrop, F Nitrofurantoina, FOS Fosfomicina, TOB Tobramicin, NET Netilmicin, AK Amikacin, CN Gentamicina

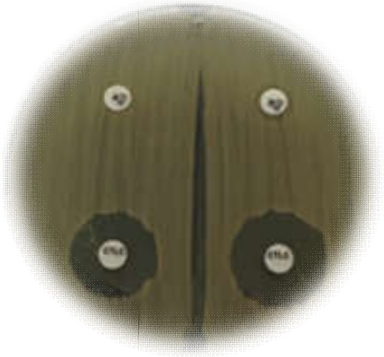
***Klebsiella Pneuminiae*-Antimicrobial susceptibility testing**

ESBL screening and confirmation by phenotypic methods

The isolates showing resistance to one or more 3rd generation cephalosporins were tested for ESBL production by the double disk diffusion test (DDDT) using three pairs of cephalosporins and their combinations with clavulanic acid (ESBL set, Mast Diagnostic). ESBL set contains 3 paired sets of cephalosporins (ceftazidime-CAZ; cefotaxime-CTX and cefpodoxime-CPD) and cephalosporins with clavulanic acid-CA (CAZ + CA; CTX + CA and CPD + CA). The inoculum and incubation conditions were the same as for standard disk diffusion recommendations. A > 5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL positive.

Detection of ESBL Producing Isolates

The modified double-disc synergy test (m-DDST) was used to detect the extended spectrum β-lactamase-producing isolates, aztreonam, ceftazidime, cefotaxime and ceftriaxone discs (30 mg) were placed around an amoxicillin-clavulanic acid disc (10 mg) at interdisc distances (centre to centre) of 20 mm on Muller-Hinton agar inoculated by bacterial suspension equal to 0.5 McFarland, a clear extension of the edge of the aztreonam, ceftazidime, cefotaxime and ceftriaxone discs inhibition zone towards the disc containing clavulanic acid was interpreted as positive for ESBL production Picture 3 (Bradford, 2001).



Picture 3. Detection of ESBL Producing Isolates

ESBL E-test

All the isolates positive by ESBL set were further confirmed by the ESBL E-test method (BioMerieux) using strips impregnated with a gradient of different concentrations (0.5–32 μg/ml) of CAZ on one side and on other side different concentration of CAZ (0.064–4 μg/ml) along with a fixed concentration of clavulanic acid (4 μg/ml). The other strip was with gradient of concentration (0.25–16 μg/ml) of CTX at one side and at the other side different concentration of CTX (0.016–1 μg/ml) with a fixed concentration of clavulanic acid (4 μg/ml). The presence of ESBL was confirmed by the appearance of a phantom zone or when CAZ MIC and CTX MIC are reduced > 3 log2 dilutions in the presence of clavulanic acid as per manufacturer guidelines. *K. pneumoniae* ATCC 700603 was used as a positive control (presence of blaSHV gene) and Escherichia coli ATCC 25922 was used as a negative control (obtained from American Type Culture Collection, USA), for both phenotypic methods and for multiplex PCR.

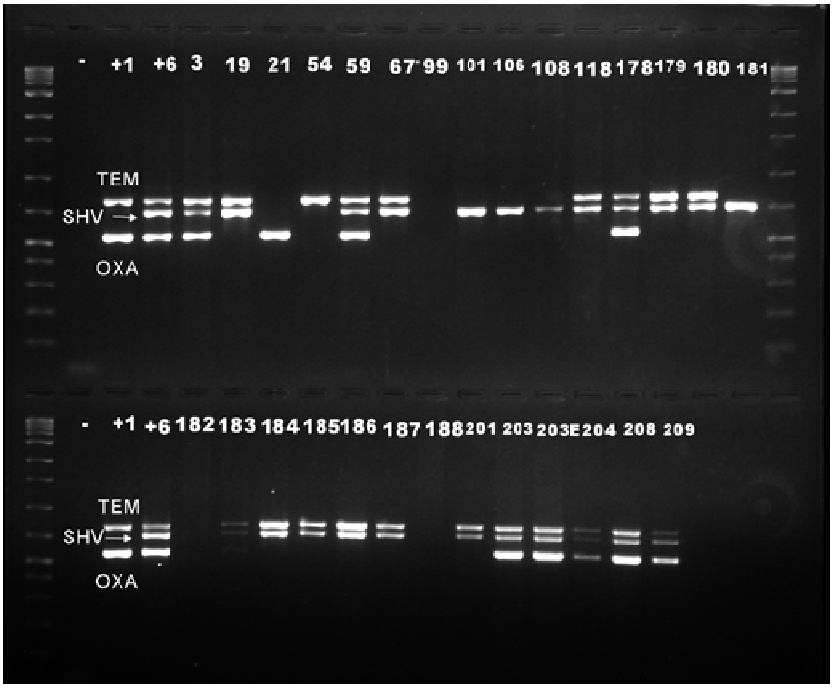
Picture 4. E-Test

PCR Detection of β-lactamases

Polymerase chain reaction technique has been used to amplify genes encoding the CTX-M β-lactamases from genomic DNA of all *Klebsiella pneumonia*. isolates with specific forward and reverse primers (Table 1)

Table 1. Primers used for the detection of beta-lactamases frequently encountered among Gram negative pathogens

Gene		Primers (5'- 3')	Reference	
bla_{TEM}	multiplex	5′-CATTTCCGTGTCGCCCTTATTC-3′	Giraud-Morin, 2003	Initialdenaturationat94єC7min; denaturation at94єC-50s, annealingat57єC-40s elongationat72єC-1m, repeated for 30 cycles; Finalextensionat72єC-7 minutes
		5′-CGTTCATCCATAGTTGCCTGAC-3′		
bla_{SHV}		5′-AGCCGCTTGAGCAAATTAAAC-3′	Poole, 2004	
		5′- ATCCCGCAGATAAATCACCAC-3′		
bla_{OXA}		5′-GGCACCAGATTCAACTTTCAAG-3′	Gupta, 2007	
		5′-GACCCCAAGTTTCCTGTAAGTG-3′		
		5′- AACCCACGATGTGGGTAGC-3′		



Picture 6. Multiplex PCR amplification_ - negative control, +1,+6 -positive control of blaTEM, blaSHV, blaOXA genes of *Klebsiella pneumonia* isolates

*gene*Amplification reactions were performed in a 25 µl volume containing 2.5 µl of 10X PCR reaction buffer with a MgCl2 (15 mM), 0.5 µl (200 µM) deoxynucleoside triphosphates mix (dNTPs, 10 mM), 1 µl (each) primers (10 pm/µl) with 0,5 µl (5 U/µl) Taq DNA polymerase. Five microlitres of the template DNA preparation was added to the reaction mixture.Both assays used identical cycling conditions. Reactions were performed in a DNA thermal cycler under the following conditions: denaturation at 94o C for 7 min followed by 30 cycles at 94o C for 50s, 57o C for 40 sec. and 72o C for 1 min and a final extension of 72o C for 7 min. After PCR amplification, 10 µl of each reaction was separated by electrophoresis in 1.5% agarose gel. Both assay products were electrophoreseed for 1 hour at 100 V in 0,5 × TBE buffer. DNA was stained with ethidium bromide (1 µg/ml) and the gels were imaged under UV light. PCR amplicon size was calculated by comparison to molecular weight size marker (1000 bp DNA ladder). All of the Set I and SET II assays produced single or multiplexed products of the predicted sizes. Methods have been described previously (Al-Agamy, 2009; Kim, 2009; Colom*et al*., 2003; Jemima*et al*., 2008).

Types TEM β-lactumases (class A)

TEM-1 is the most commonly-encounteredβ-lactumase in Gram-negative bacteria. Up to 90% of ampicillin resistance in E.coli is due to the production of TEM-1.

Also responsible for the ampicillin and penicillin resistance that is seen in H.influenzaeand N.gonorrhoeae in increasing numbers. Although TEM-type β-lactumasesare most often found in *K.pneumoniae*, they are also found in other species of Gram-negative bacteria with increasing frequency.

The amino acid substitutions responsible for the ESBL phenotype cluster around the active site of the enzyme and change its configuration, allowing access to oxyimino-β-lactum substrates.

Opening the active site to β-lactum substrates also typically enhances the susceptibility of the enzyme to b-lactumase inhibitors, such as clavulanic acid. Single amino acid substitutions at positions 104, 164, 238, and 240 produce the ESBL. Phenotype, but ESBLs with the broadest spectrum

usually have more than a single amino acid substitution. Based upon different combinations of changes, currently 140 TEM-type enzymes have been described. TEM-10, TEM-12, and TEM-26 are among the most common in the United States. SHV β-lactumases (class A) SHV-1 shares 68 percent of its amino acids with TEM-1 and has a similar overall structure. The SHV-1 β-lactumaseis most commonly found in K. pneumoniae and is responsible for up to 20% of the plasmid mediated ampicillin resistance in this species. ESBLs in this family also have amino acid changes around the active site, most commonly at positions 238 or 238 and 240. More than 60 SHV varieties are known. They are the predominant ESBL type in Europe and the United States and are found worldwide. SHV-5 and SHV-12 are among the most common.

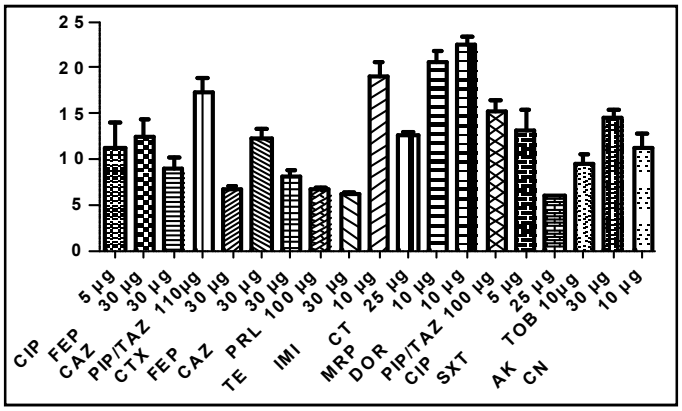
Table 2. *Klebsiella pneumonia*-SampleswhichcontainblaTEM, blaSHVandblaOXA

Sample	Gene TEM,SHV,OXA		
3	TEM	SHV	OXA
19	TEM	SHV	
21			OXA
54	TEM		
59	TEM	SHV	OXA
67	TEM	SHV	
99			
101			
106		SHV	
108		SHV	
118	TEM	SHV	
178	TEM	SHV	OXA
179	TEM	SHV	
180	TEM	SHV	
181		SHV	
182			
183	TEM	SHV	
184	TEM	SHV	
185	TEM	SHV	
186	TEM	SHV	
187	TEM	SHV	
188			
201	TEM	SHV	
203	TEM	SHV	OXA
203E	TEM	SHV	OXA
204	TEM	SHV	OXA
208	TEM	SHV	OXA
209	TEM	SHV	OXA

OXA β-lactumases (class D)

OXA β-lactumaseswere long recognized as a less common but also plasmid-mediated β-lactumase variety that could hydrolyze oxacillin and related anti-staphylococcal Penicillins. These β-lactumases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXA-type β-lactumases confer resistance to Ampicillin and Cephalothin and are characterized by their high hydrolytic activity against Oxacillin and Cloxacillin and the fact that they are poorly inhibited by clavulanic acid. Aminoacid substitutions in OXA enzymes can also give the ESBL phenotype.

Also for Determining the quality of the resistance Klebsiellapneumoniae We also used statistical analysis GraphPad, which showed interesting results.(P value-< 0,0001).



Kruskal-Wallistest	Klebsiellapneumoniae
P value	< 0,0001
Exactorapproximate P value?	GaussianApproximation
P valuesummary	***
Do the medians vary signif.(P < 0.05)	Yes
Numberofgroups	19
Kruskal-Wallisstatistic	179,5

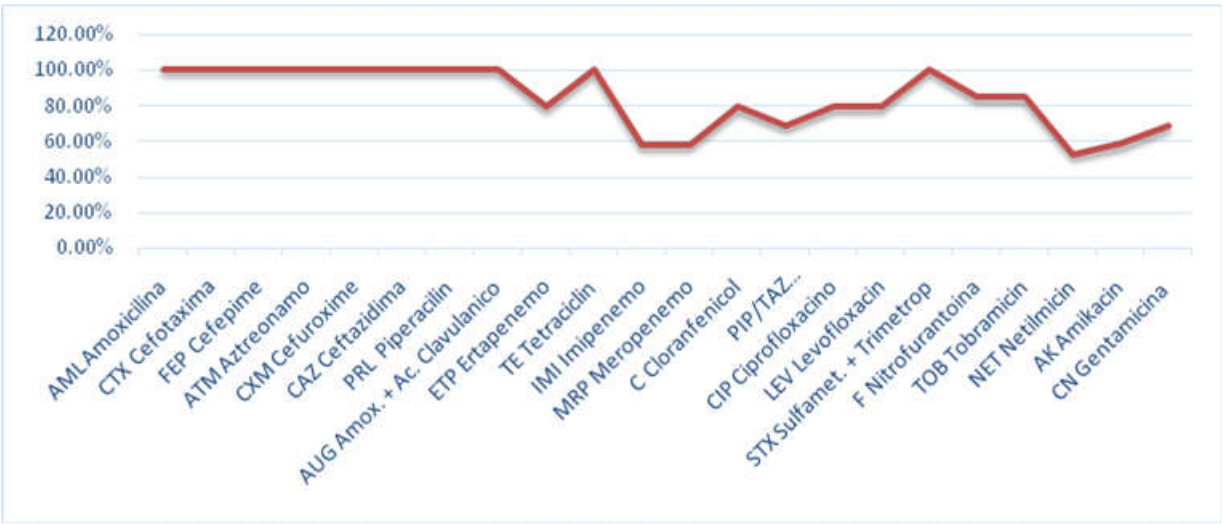


Figure 1. Antibiotic Resistance profile *Klebsiella pneumonia*

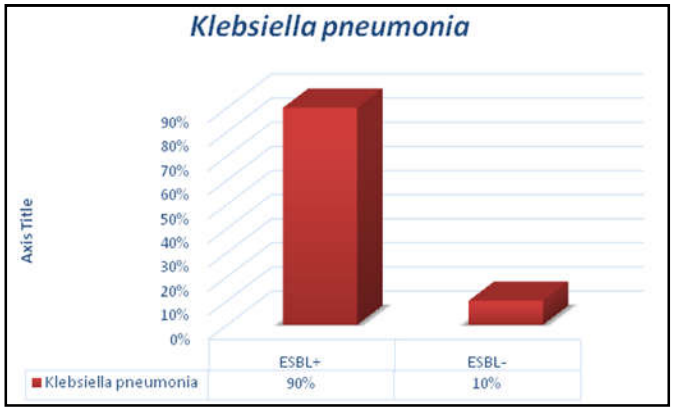


Figure 2. ESBL – positive strains out of a total number *Klebsiella pneumonia*

RESULTS

Twenty-eight *Klebsiella pneumonia* producing TEM, SHV, OXAgene isolates were detected in different biological samples, namely in sputum (n=10), urine (n=8) and abdominal fluid (n=8), collected in different hospital services. The infection isolates showed an extended resistance profile to aminoglycosides, fluoroquinolones and tetracycline. isolates showed specific amplification for bla_{TEM}, bla_{OXA}, bla_{SHV}, families. Asthediagram shows all sample was resistance the antibiotics *AML, CTX, FEP, ATM, CXM, CAZ, PRL, AUG, TE, STX,* Also quite high rateofresistance the antibiotics *ETP,C, PIP, CIP, LEV, F, DOR, MRP, TGC, IMI, NET, AK,CN.* Relatively sensitive antibiotics was *FOX in our samples were found broad spectrum betalactamase form. 90% Samples ESBL positiveand 10% ESBL negative, which Showedphenotypic assay ESBL confirmationtests* (Figure 2).

DISCUSSION

In recent years, the problem of increasing resistance to antibiotics has threatened the entire world. Production of beta-lactamase, which hydrolyses and inactivates beta-lactam antibiotics, has been one of the most important resistance. Mechanisms of many bacterial species, mainly in the Enterobacteriaceae family. Resistance to an extended spectrum beta-lactams among gram-negative pathogens is increasingly associated with ESBLs. *K. Pneumoniae* is the most prevalent among ESBL-producing microorganisms, confirming international multicentric studies (Bali*et al*., 2010; Jemima *et al*., 2008; Luzzaro *et al*., 2006; Goyal, 2016)

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