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Screening of Multi-Drug Resistant (MDR) β-Lactamase Producing *Klebsiella Pneumoniae* and *Klebsiella Oxytoca* from Clinical Isolates

Saroj Kumar Sah[1]
Assistant professor, Department of Biomedical Engineering, Excel Engineering College , Komarapalayam, TN-India

Dr. S. Hemalatha[2]
Professor and Dean, School of Life sciences, B.S. Abdur Rahman, Crescent Institute of Science and Technology, Chennai, India

Abstract

Multidrug resistant β-lactamase producing bacteria are a great threat in the clinical practice as they pose a hindrance in treating various infections. The two isolates studied here cause many deadly diseases and in order to provide a solution to this problem; the antibiotic profiling is important which can present an idea on the proper use of antibiotics in treating the infections. A total of 50 clinical isolates of *Klebsiella* (38 *Klebsiella pneumoniae* and 12 *Klebsiella oxytoca*) were subjected to phenotypic confirmatory test for extended-spectrum beta-lactamase (ESBL) production. The ESBL test revealed that 90 % (45/50) of the isolates were resistant to at least one of the 3rd generation cephalosporins. Out of those isolates, 64% (28/45) were *Klebsiella pneumoniae* and 36% (16/45) were *Klebsiella oxytoca*. Susceptibility testing revealed that most of the isolates showed multi-drug resistance. Biofilm formation was carried out using congo red agar medium and the positive biofilm producers were observed as dark colonies with crystalline constancy. Non-biofilm producers were observed as pink colonies with infrequent dark spots. Molecular studies on the detection of bla_{SHV} and AmpC genes, by conventional Polymerase Chain Reaction (PCR), revealed that out of 73.0% (33/45) of phenotypically ESBL positive strains, 54.5% (18/33) were positive for AmpC and 60.6 % (20/33) for bla_{SHV} genes.This study presents a simple and accurate method to easily carry out the laboratory tests for ESBL detection and an early detection of infections by ESBL positive organisms allows rapid initiation of therapeutic methods to treat those infections and reduce the use of antibiotics.

Keywords: ESBL, Multi-drug resistance, AmpC, bla_{SHV}

Background

The genus *Klebsiella* belongs to the family *Enterobacteriaceae* and live in freshwater environments including shallow water, sewage and soil, as well as mammalian mucosal surfaces. *Klebsiella pneumoniae* and *K. oxytoca* are responsible for nosocomial infections including urinary tract infections, pyometra, upper respiratory infections and septicemia in human beings and various partner animals [Kazuki et al., 2016[1]. Gram negative pathogens of the *Enterobacteriaceae* have developed various resistance mechanisms to overcome the toxic effect of antibiotics [Ramanpreet et al., 2014[2]. Extended-spectrum beta-lactamases (ESBLs) and Metallo beta-lactamases (MBLs) are powerful enzymes produced by *Enterobacteriaceae* hydrolyzing beta-lactam antibiotics resulting in resistance of *Enterobacteriaceae* towards antibiotics [Mariateresa et al., 2015[3]. The ESBL carbapenemase producing *Enterobaceriaceae* (CPE) are a serious threat worldwide mostly in hospital acquired infections. The alarming rate with which CPE are spreading is a very serious issue because carbapenems are the last option in treating infections caused by multi-drug resistant bacteria [Nadezhda et al., 2015[4]. Amongst Gram-negative organisms, *Klebsiella pneumoniae* plays a major role in nosocomial infections and stands as the third most common Gram-negative bacteria, after *P. aeruginosa* and *A. baumannii* in causing nosocomial infections. *Klebsiella pneumoniae* can thrive in hospital environments and has the ability to colonize human skin facilitating its transmission among patients. However, the emergence of antibiotic resistant *Klebsiella pneumoniae* is due to inappropriate and over-use of antibiotics. The major mechanism for resistance towards beta-lactam antibiotics involves production of beta-lactamases such as AmpC beta-lactamases (AmpC) and extended-spectrum beta-lactamases (ESBLs). AmpC beta-lactamases come under group I cephalosporinases and have the potential to confer resistance to a diverse range of beta-lactam antibiotics that include third generation cephalosporins and aztreonam. AmpC together with porin loss can act as intermediate agents in facilitating resistance to carbapenems [Leila et al., 2015[5]. Plasmid mediated AmpC beta-lactamases have emerged as a result of chromosome to plasmid transfer, which can promote spread between bacterial communities including *Klebsiella* spp. which typically do not possess chromosomal AmpC [Subha et al., 2003[6]. Biofilm is an aggreate of Gram positive and Gram negative bacteria where the bacterial cells get attached towards each other on the surface. The attached cells in the biofilm are embedded in the matrix of extracellular polymeric substance (EPS), which is produced by the attached cells in the biofilm. The formation of biofilm by the bacteria protects them against various antimicrobial agents and provides them with extra resistance (Zuberi and Nadim, 2017)[7]. *Klebsiella pneumoniae* has the

capability of biofilm formation and is responsible for causing infections in immunocomparmised patients (Haili et al., 2016)[8].

The main purpose of this study was to determine the percentage of ESBL producing isolates of *Klebsiella pneumoniae* and *Klebsiella oxytoca* from various clinical specimens. *Klebsiella* strains were also assayed for their biofilm forming capability on congo red agar medium. Molecular detection of AmpC and bla_{SHV} genes involved in the production of ESBL in the clinical isolates of *Klebsiella* was carried out.

Materials and Methods

Ethical approval

The ethical clearance was obtained from Institutional Ethics Committee, School of Life Sciences, B. S. Abdur Rahman University (Ref. no. BSAU: REG-OFF: 2016/02SLS), Vandalur, Chennai, India. The main purpose of performing this study was for the standard care and the need for informed consent was not necessary. The identity and all the personal information about the patients was confidential.

Bacterial strains collection and identification

This study was conducted from August to November 2016 at School of Life Sciences, B.S.A. Crescent Institute of Science and Technology, Chennai. A total of 50 *Klebsiella* spp. were collected from the Department of Microbiology, Tagore Medical College and Hospital, Tamilnadu, India. The clinical isolates were identified as *Klebsiella pneumoniae* (n=38) and *Klebsiella oxytoca* (n=12). The source of these isolates was Urine (n=34) followed by Sputum (n=7), wound swab (n=2), pus (n=2), blood (n=1), throat secretion (n=1), esophageal feeding tube (n=1) and hand wash (n=2). Out of 50 clinical isolates, 23 were from male patients and 27 females. All clinical strains were identified by standard conventional biochemical techniques (Farmer, 2003)[9] and were stored at -80°C in 15% glycerol for further use.

Confirmatory test for ESBLs production

Phenotypic ESBL confirmatory tests were performed as per [CLSI 2013[10] recommendations using quality control standard strains of *Klebsiella pneumoniae* (ATCC 700603) (Beta-lactamase positive) and *Escherichia coli (*ATCC 25922) (Beta-lactamases negative). All isolates were subjected to an ESBL confirmatory test on the basis of preliminary screening. A double disk diffusion test was performed by placing an antibiotic disk of ceftazidime (CAZ 30 µg) versus ceftazidime/ clavulanic acid (CAZ/CA 30/10 µg), cefotaxime (CTX 30 µg) versus cefotaxime/clavulanic acid (CTX/CA 30/10 µg), cefepime (CPM 30 µg) versus cefepime/ clavulanic acid

(CPM/CA 30/10) on Mueller-Hinton plates spread with the test strain. The interpretation of test results was recorded by following CLSI guidelines. An approximate increase of greater than or equal to 5 mm in efficacy for the disks containing antibiotics and inhibitors (CTX/CA, CAZ/CA, CPM/CA, AMC/CA) versus the comparative antibiotics alone (CTX, CAZ, AMC) was considered as ESBL positive.

Antibiotic susceptibility testing

The ESBL producing isolates were subjected to antimicrobial susceptibility testing by disk diffusion [Bauer et al., 1965[11]. These antibiotic discs were employed: ampicillin (10 µg), cefpodoxime (30 µg), cefazolin (30 µg), cefotetan (30 µg), aztreonam (10 µg), levofloxacin (5 µg), meropenem (10 µg), imipenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), cefotaxime (30, µg) ceftazidime (30, µg), cefepime (30, µg) and amoxicillin (20, µg), The Minimum Inhibitory Concentration (MIC) for ESBL producing *Klebsiella* spp. were determined using Ezy MIC™ paper strips as per CLSI guidelines. The Ezy MIC™ strips coated with cefotaxime, ceftazidime, and cefepime, in the range of 0.016-256 µg/ml, were placed on Muller Hinton agar plates seeded with the test strain. *E. coli* (ATCC 25922) was used as a reference strain.

Biofilm characterization

Congo red agar medium was prepared. The medium consisted of brain heart infusion broth (Hi-Media) 37.5 g/l, Sucrose 50 g/l, agar 15 g/l and Congo red 0.8 g/l (SRL). A concentration aqueous solution of Congo red stain was prepared and autoclaved (121 °C for 15 min). Congo red stain was separately autoclaved from other media components and was added to agar after it had cooled down (55^0C). The strains were inoculated in the medium and incubated (aerobically) at 37^0C for 24-48 hours (Freeman et al., 1989)[12]. Biofilm production was observed by the formation of black colonies with dry crystalline constancy. Non-biofilm producers appeared as smooth and pink colonies.

Molecular characterization

PCR for bla_{SHV}, and AmpC

Phenotypic ESBL positive strains were subjected to molecular screening for detection of bla_{SHV}, The AmpC gene was detected by conventional Polymerase Chain Reaction (PCR) with some modifications [Hijazi et al.,2016[13]. Crude genomic DNA was prepared from an overnight culture on an LB agar plate. A single colony was suspended on 100 µl of ultra-purified water. The colony suspension was lysed

by heating at 95±2°C for 5 min. Lysed cells were centrifuged at 12000 RPM for 5 minutes. The supernatant was used as template DNA for amplification. ESBL producing isolates were amplified using AmpC and bla_{SHV}gene primers. For AmpC, the forward primer was 5′-ATTCCGGGTATGGCCGT-3 and the reverse was 5′-GGGTTTACCTCAACGGC-3′ [Jones, 2010[14]. For the detection of bla_{SHV} gene, the forward primer was 5′-ACCATGAGCGATAACAGCG-3′ and the reverse was 5′-TCATTCAGTTCCGTTTCCCAG-3′. The PCR primers were purchased from IDT India. The amplification reactions were performed on a Mastercycler nexus gradient (Eppendorf USA). The reaction mixture comprised of 5 µl of PCR master mix 2X (Amplicon III) reaction buffer containing (0.11 units/µl Taq DNA polymerase, 1.65 mM $MgCl_2$, 0.22 mM of each dNTP) 2 µl forward and reverse primers encoding blaSHV SHV and AmpC 3 µl of template DNA in a final volume of 10 µl. The amplification reactions were performed in a DNA thermal cycler under the following conditions: Initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for1min, annealing at 58°C for AmpC and 59°C for SHV extension at 72°C for 45s and the final extension of 72°C for 5 min. The PCR products, 3 µl of each reaction, were analyzed by electrophoresis in 1.5 % w/v agarose gel at 80 V for 45 min in1X TAE buffer with a 100 bp DNA ladder (Gene Direx). The DNA was stained by using ethidium bromide (1 µg/ml) and images were taken under ChemiDoc MP System (Bio-Rad, USA 2013).

Results

Antibiotic Susceptibility Testing

The percentage of resistance todifferent antibiotic formulations was observed as follows: Ampicillin (100%), Amoxicillin (100%), Ceftazidime (96%), Cefotaxime (88%), Cefepime (64%), Cefpodoxime (35%), Cefotetan (44%), Ciprofloxacin (44%), Levofloxacin (44%), Gentamicin (42%), Aztreonam (34%), Meropenem (52%) and Imipenem (14%).These results reveal Multi drug resistance (MDR) of the isolates towards the above antibiotics (Figure 1). A total 86% of clinical isolates were sensitive to imipenem, 58% to Gentamicin, 48% to Meropenem, 66% to Aztreonam, 56% to levofloxacin, 56% to Ciprofloxacin, 56% to Cefotetan, 65% to Cefpodoxime, 36% to cefepime, 12% to Cefotaxime and 4% to ceftazidime.

Phenotypic ESBL Detection

Ninety percent (45/50) of the isolates displayed resistance to at least one of the 3rd generation cephalosporins. Sixty four percent (28/45) of those isolates were *Klebsiella pneumoniae* and the rest, 36% (16/45), were *Klebsiella oxytoca.* A total of 50 clinical isolates displayed resistance towards ampicillin and amoxicillin. Following CLSI guidelines, the phenotypic confirmatory test was performed for ESBL

detection and 45 isolates showed enhanced susceptibility to cefotaxime, ceftazidime and cefepime in the presence of clavulanic acid, confirming ESBL production. Very few isolates treated with a combination of AMX / (AMX+CA) showed complete resistance towards both the antibiotic formulations (Figure 1b-1e) may be overexpression of ampicillinase.

Biofilm characterization on Congo red Agar

Out of the 50 clinical isolates, 33/50 (56%) showed the biofilm production on CRA medium. Positive biofilm formation result is shown as black colonies with dry crystalline consistency in Fig. 2a and 2c. Non-slime producers usually remained pink with infrequent darkness at the center of colonies (Fig, 2b). Out of 50 isolates, 11 were strong biofilm producers, 14 moderate, 8 weak and 17 isolates did not show any biofilm formation.

Table 1-Total number of *Klebsiella* spp. isolated from different specimens.

S. No.	Source of the isolate	Total number of *Klebsiella* spp. isolated	No. of ESBL producers
01	Urine	34	32
02	Sputum	7	4
03	Wound swab	2	2
04	Pus	2	2
05	Blood	1	1
06	Throat secretion	1	1
07	E. Tube	1	1
08	Hand wash	2	2
Total number		50	45(90%)

Molecular analysis of AmpC and bla$_{SHV}$

Molecular analysis of gene expression encoding AmpC and bla$_{SHV}$ through polymerase chain reaction (PCR) showed that 75.0% (33/45) of phenotypically ESBL positive strains 40% (18/33) were positive for the AmpC gene and 60 % (20/33) were positive for bla$_{SHV}$gene. The amplified products of AmpC and bla$_{SHV}$ are shown in Fig 3.

Discussion

ESBL production by gram-negative bacteria has been a major hurdle in treating the infections caused by these bacteria [Stürenburg et al., 2003; Bradford, 2001[15, 16]. Initially, until 1990, ESBL production was confined to *E. coli* and *Klebsiella* species and to some geographical regions. However, in the late 1990s, ESBL producing bacteria have been spreading to other genera of bacteria such as *Enterobacter* and *Proteus* [Coudron et al., 2002; Spanu et al., 2002; Albertini et al., 1997; Bonnet et. al.,1999[17-20]. Apart from diverse growth of ESBL producing strains, ESBL phenotypes have developed into more complex forms due to multiple-enzyme production such as various ESBL variants that are resistant to inhibitors, dispersal of CTX-M types, plasmid-mediated AmpC, ESBL production in AmpC-producing strains, hyperproduction of enzymes and porin loss. The main possible reason that could have resulted in uncontrolled spread of ESBL producing bacteria and the associated treatment failures might have been the failure to find complex resistance phenotypes by physicians or any laboratory procedures [Stürenburg et al., 2004[21]. This particular study dealt with clinical isolates of *Klebsiella pneumoniae* (n=38) and *Klebsiella oxytoca* (n=12) that were recovered from urine (n=34), Sputum (n=7), wound swab (n=2), pus (n=2), blood (n=1), throat secretion (n=1), E. tube (n=1) and hand wash (n=2). This study presented phenotypic confirmation of ESBL positive isolates of *Klebsiella pneumoniae* and *K. oxytoca* following CLSI guidelines using a double disk diffusion test involving ceftazidime (CAZ 30 µg) versus ceftazidime/ clavulanic acid (CAZ/CA 30/10 µg), cefotaxime (CTX 30 µg) versus cefotaxime/clavulanic acid (CTX/CA 30/10 µg) and cefepime (CPM 30 µg) versus cefepime/ clavulanic acid (CPM/CA 30/10) antibiotic discs. Previously, different studies have been carried out regarding the percentage of ESBL production such as the one carried out by Kaur and Kaur (2017) which reported 33.3 % of ESBL producers from Gram-negative isolates obtained from burn infections where 14 % of the isolates produced inducible AmpC beta lactamases. According to this study, 26.3 % of *Klebsialla pneumoniae* strains were ESBL positive apart from *E. coli* (31.5 %) and *P. aeriginosa* (21 %) (Kaur and Kaur, 2017)[22]. Other studies by Shukla *et al*. (2004) and Taneja *et al*. (2008) have reported 30.18% and 36.5% of ESBL producers respectively [Shukla *et al*. 2004; Taneja *et al*. 2008[23, 24]. Our study presented a higher percentage of ESBL producing strains (90%) compared to these studies, where 64% (28/45) of the isolates were *Klebsiella pneumoniae* and the rest 36% (16/45) were *Klebsiella oxytoca.* A study carried out by Bülüç *et al*. (2003) reported somewhat similar percentages of ESBL producing *Klebsiella* spp including 48% of *Klebsiella pneumoniae* and 40% *K. oxytoca* [Bülüç et al.,2003[25]. Two other studies in Pakistan and Singapore have reported a similar percentage of *Klebsiella pneumoniae* producing ESBL isolates. A study carried out by Ullah *et al*. (2009)

reported 58.7% and Chlebicki *et al*. (2004) reported 44% of *Klebsiella pneumoniae* isolates respectively [Ullah *et al*. 2009; Chlebicki *et al*. 2004[26, 27]. Out of 50 clinical isolates, 90 % were ESBL positive by phenotypic analysis whereas the molecular data showed that 75 % of the isolates were ESBL positive with 40 % of those positive for the AmpC gene and 60 % positive for the bla_{SHV} gene. Molecular studies on SHV and AmpC genes have been previously carried out by various researchers such as the one reported by Bali *et al*. (2010), where 14.89 % of ESBL isolates were positive for the SHV gene [Bali *et al*. 2010[28]. SHV beta lactamases are mostly found in *E. coli* and *Klebsiella pneumoniae* but can be present in other members of *Enterobacteriaaceae.* AmpC production has been reported in various *Klebsiella pneumoniae* isolates For example, 8 % of *Klebsiella pneumoniae*isolated from blood culture were positive for AmpC [Sari et al.,2013[29]. 12 % of *Klebsiella pneumoniae* isolates were positive for AmpC in a study carried out in Pakistan [Shafiq et. al., 2013[30]. 13.6 % of *Klebsiella* spp. were positive for AmpC as reported by Oberoi *et al*. (2013)[31] . In *Enterobacteriaeceae,* AmpC is originally chromosomally-mediated but plasmid-mediated isolates have arose that contain no chromosomal [Shayan et. al., 2014[32]. We have documented 40% (18/33) of the *Klebsiella* isolates positive for AmpC gene and 60 % (20/33) positive for bla_{SHV} gene and that percentage is a lot higher when compared to the above mentioned studies.

Conclusions

This study provides an insight to the higher percentage of ESBL producers in the clinical isolates of *Klebsiella.* From phenotypic confirmatory tests, clinical isolates of *Klebsiella* showed a higher percentage of ESBL production compared to MBL production. Though the phenotypic measurements are not sensitive enough but still they are economical and do not require expertise and professionals and can be performed easily. The results of this study demonstrate the serious threat that the spread of ESBL and MBL producing isolates of *Klebsiella* poses*,* hence early detection of infection by resistant strains can help in providing relief to patients and can reduce mortality rates.

Abbreviations

ESBL: Extended-spectrum beta-lactamase; MBLs: Metallo-beta-lactamases; EDTA: Ethylenediaminetetraacetic acid; DDST: Double disc synergy test; CPE: Carbapenemase producing *Enterobaceriaceae*; CLSI: Clinical laboratory standards institute; PCR: Polymerase chain reaction; ATCC: American type culture collection; AMP: ampicillin; AMX: amoxicillin; CAZ: ceftazidime; CTX: cefotaxime; CPM: cefepime; CPD: cefpodoxime; CTN: cefotetan; CIP: ciprofloxacin; LV: levofloxacin; GEN: gentamicin; AT: aztreonam; MRP: meropenem; IPM: imipenem; AMC:

amoxyclav; CA: clavulanic acid; CAZ/CA: ceftazidime/clavulanic acid; SHV: Sulfhydryl variable; VIM: Verona integron-encoded metallo-beta-lactamase; AmpC: Ampicillinase; MIC: Minimum inhibitory concentration; TAE: Tris-acetate; UTI: Urinary tract infection

Declarations

Ethics approval and consent to participate

ESBL strains of *Klebsiella* were obtained from Tagore Medical College, Chennai, India after proper approval from BS Abdur Rahman University ethical committee (Ref. no. BSAU: REG-OFF: 2016/02SLS). The informed consent was not compulsory as the isolates used in this study were obtained as a part of standard care. The identity and all the personal data about the patients was confidential.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information files

Competing interests

The authors declare that they have no competing interests

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Author's contributions

SKS designed and performed the experiments, analyzed and interpreted the data. UR drafted the manuscript and critically revised it. NB approved the final version of the manuscript to be published. SB provided the isolates used for this study with all the details and approved the final version of the manuscript to be published. HS designed the whole idea behind this work, critically revised the manuscript and approved the final version of the manuscript to be published.

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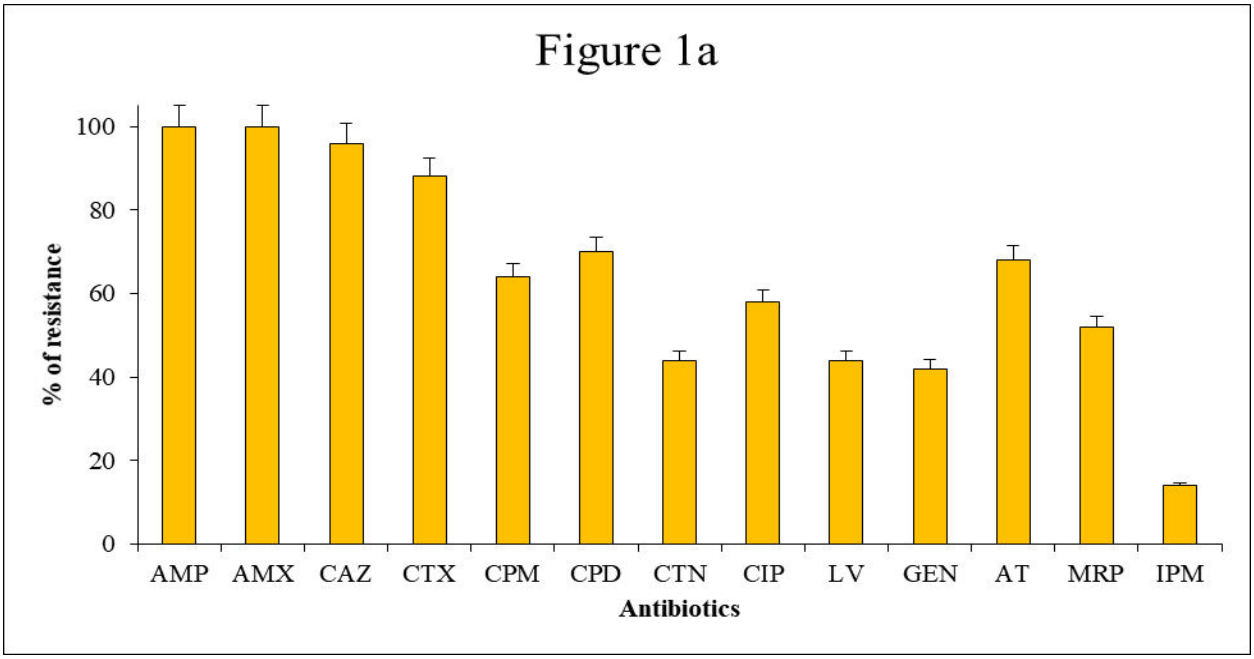
Figure legends

Figure 1: (a) Percentage of resistance of *Klebsiella* species towards different antibiotics **(1b-1e)** ESBL producing *Klebsiella* spp. (a) Cephotaxime (CTX 30 µg) versus Cephotaxime/Clavulanic acid (CTX+CA, 30/10 µg), (b) Ceftazidime (CAZ 30 µg) versus Ceftazidime/Clavulanic acid (CAZ+CA, 30/10 µg) (c) Cefepime (CPM 30 µg) versus Cefepime/Clavulanic acid (CPM+CA, 30/10 µg), (d) Amoxicillin (AMX 20µg) verses Amoxicillin/Clavulanic acid (AMX+CA 20/10 µg)

Figure 3: (a) PCR product of AmpC gene from *Klebsiella* isolates separated on agarose gel (1.5 %) for detection of AmpC gene (M = 100 bp DNA marker, Lane 1, 2 =ESBL Positive strains, Lane 3 = Positive control for AmpC, N =Negative control for AmpC) **(b)** PCR product of AmpC gene from *Klebsiella* isolates are separated on agarose gel (1.5 %) for detection of bla_{SHV} gene (M = 100 bp DNA size marker, Lane 1-3 =ESBL Positive strains, Lane N = Negative control for bla_{SHV}, Lane 4 = Positive control for bla_{SHV} gene)



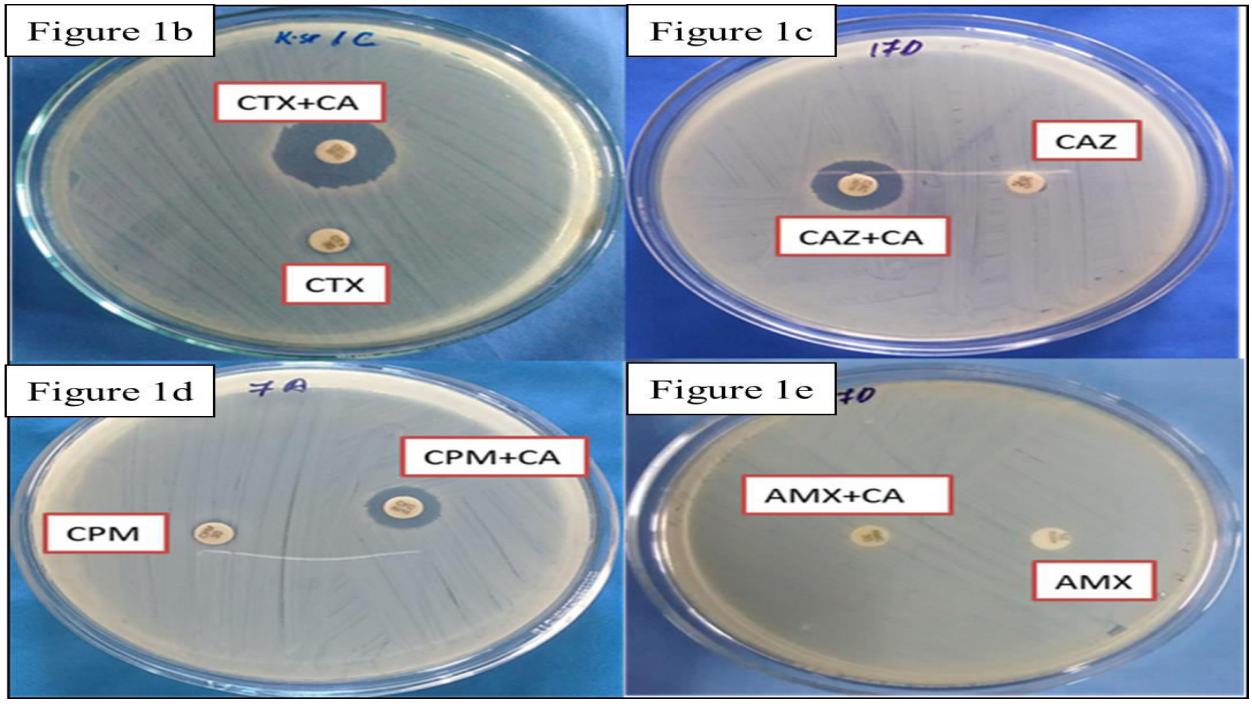
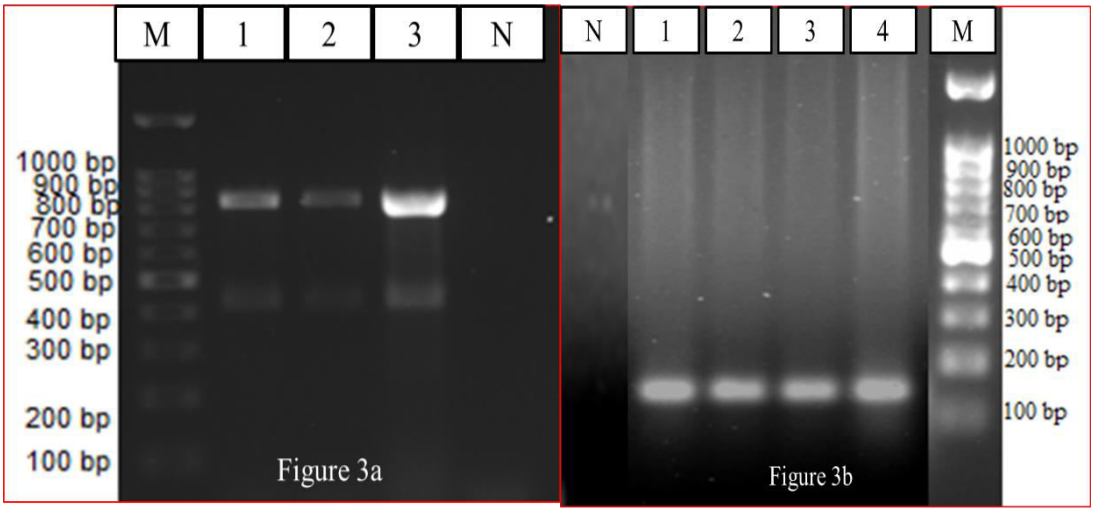


Figure 1b
CTX+CA
CTX
Figure 1c
CAZ
CAZ+CA
Figure 1d
CPM+CA
CPM
Figure 1e
AMX+CA
AMX


M
1
2
3
N
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
Figure 3a
N
1
2
3
4
M
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
Figure 3b