Biomedical Current Insights
DOI: https://doi.org/10.63626/nmd11308
ISSN Online: 3104-414X
ISSN Print: XXXX-XXXX

Research Article

Biochemical and molecular evidence of β-lactamases in klebsiella species isolated from septic children admitted in a tertiary care hospital

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ABSTRACT
Background: Klebsiella is a genus of Gram-negative rod-shaped bacteria with a prominent polysaccharide-based capsule. Antibiotic resistance patterns of Klebsiella species are often associated with molecular features. Purpose of this study was to detect Extended-Spectrum Beta-Lactamases (ESBL), AmpC Beta-Lactamases (AmpC) and Metallo-Beta-Lactamases (MBL) enzymes in Klebsiella species.
Methods: Total 259 samples were collected from patients (≤15 years old) admitted in various wards and visiting outpatient department of The Children Hospital, Lahore. All samples were processed and screened for ESBL, MBL and AmpC enzymes. Molecular detection by PCR was done to detect their genotypes and results were analyzed using SPSS software version 25.
Results: Out of 259 patients, ESBL positive, MBL positive and AmpC positive were 53 (20.5%), 64 (24.7%) and 86 (33.2%), respectively. The isolates found positive to screening test were further processed for molecular analyses. The results of the PCR amplification of the genes showed the presence of NDM-1 in 59 (22.8%), CIT in 30 (11.6%), SHV in 22 (8.5%), CTX-M in 14 (5.4%), EBC gene in 12 (4.6%) and TEM in 2 (0.8%) isolates. Two isolates co-produced all the three enzymes followed by 9 that co-produced MBL and ESBL, 4 isolates co-produced AmpC and MBL and 4 co-produced ESBL and AmpC.
Conclusion: A large number of patients were found to have serious bacterial infections with significant number of ESBL, MBL and AmpC-production along with co-existence.
Key Words: Klebsiella, Beta-Lactamases, Occurrence

INTRODUCTION

β-lactams are widely used as antibiotics because of their comparatively high effectiveness, low cost, ease of delivery, and minimal side effects [1] Extensive and irrational use of antibiotics have led to emergence of multi-drug resistance in bacteria. Prevalence of β-lactamases, enzymes that provide antibiotic resistance, is increasingly being reported worldwide. Klebsiella spp is one of the most common β-lactamase producing bacteria causing respiratory infections and post-surgical complications in immunocompromised patients [2].

Corresponding Author: Muhammad Usman
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Received: 27.07.2025
Revised: 27.12.2025
Accepted: 28.12.2025
Published: 30.12.2025

Three major groups of β-lactamase enzymes such as cephalosporin's (AmpC), Extended Spectrum β-Lactamases (ESBL) and Carbapenemases such as Metallogen β-Lactamase (MBL) are of great concern in health care settings. The presence of ESBL, AmpC and MBL in a single isolate reduces the effectiveness of β-Lactam. β-lactamase inhibitor combinations and confer resistance to Carbapenems. Often these enzymes are co-expressed in same isolates and are responsible for resistant mechanisms. This resistance constitutes urgent threat to patient management and public health. Co-existence of different classes of β-lactamases in a single isolate may pose diagnostic challenge. Detection of these multi-drug-resistant strains may prompt the implementation of isolation procedures to prevent cross transmission to other patients [3].
Multiple drug resistance (MDR) by Klebsiella spp was first reported in United States, followed by Europe, South America, and Asia. At present, infections caused

by MDR Klebsiella spp have become a major problem, as few antibiotics are available, resulting in higher morbidity, longer hospitalization, increased mortality rates, and excessive healthcare costs compared with infections associated with antibiotic-susceptible micro-organisms [4]. However, the prevalence of antibiotic-resistant bacteria significantly varies according to region, country, and susceptible population, as seriousness of this problem is significantly associated with the measures applied to control the spread of drug-resistant bacteria [5].

Emergence of resistance to β-lactam antibiotics began even before first β-lactam, penicillin, was developed. Many genera of gram-negative bacteria possess naturally occurring, chromosomally mediated β-lactamases. These enzymes are thought to have evolved from penicillin-binding proteins, with which they show some sequence homology. Over the last 20 years, many new β-lactam antibiotics have been developed that were specifically designed to be resistant towards hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged that caused resistance to that class of drug. Selective pressure of the use and overuse of new antibiotics in the treatment of patients has been selected for new variants of β-lactamase. Because of their increased spectrum of activity, these enzymes were called extended-spectrum β-lactamases (ESBLs). Today, over 150 different ESBLs have been described [6]. A variety of β-lactamases which include ESBLs, AmpC and MBL, have emerged with the most worrisome mechanism of resistance among the gram-negative bacteria, which pose a therapeutic challenge to the health care settings [7].

Mechanism of action of β-lactam antibiotics is bactericidal. Bacteria develops resistance to antimicrobial agents according to their principle mechanisms of action that include interference with cell wall synthesis (e.g., β-lactams and glycopeptide agents), inhibition of protein synthesis (macrolides and tetracyclines), interference with nucleic acid synthesis (fluoroquinolones and rifampin), inhibition of metabolic pathway (trimethoprim-sulfamethoxazole), and disruption of bacterial membrane structure (polymyxins and daptomycin) [8, 9].

ESBL enzyme hydrolyzes Penicillin, Cephalosporins and Aztreonam and is encoded by movable genes. It plays important role in MDR gram negative bacteria. Most ESBLs can be divided into three groups: TEM, SHV, and CTX-M types. Klebsiella spp remains the major ESBL-producing organisms isolated worldwide. A recent report from the Infectious Diseases Society of America listed ESBL-producing Klebsiella spp as one of the six drug-resistant microbes to which new therapies are urgently needed. Because of the increasing importance of multidrug resistant ESBL-producing Klebsiella spp in the community, clinicians should be aware of the potential of treatment failures associated with serious infections caused by these bacteria [10].

MBL is plasmid mediated enzyme classified into three main groups. The emergence of carbapenem-resistant Enterobacteriaceae is worrisome, since antimicrobial treatment options are very restricted. Resistance to Carbapenems may involve several combined mechanisms which includes modifications to outer membrane permeability and up-regulation of efflux system associated with hyper production of AmpC β-lactamases, ESBL or production of specific carbapenem-hydrolyzing β-lactamases (Carbapenemases). Carbapenemases found in Enterobacteriaceae can be metallo-β-lactamases, expanded-spectrum oxacillinases, or clavulanic-acid inhibited β-lactamases [11].

AmpC enzymes are controlled by AmpC genes on chromosomes (individual) or plasmid (permanent type). This enzyme is widely associated with MDR phenotypes. AmpC β-lactamases are clinically important enzyme associated with resistance to a wide variety of β-lactam drugs such as Boronic acid and Cloxacillin [12]. AmpC enzymes hydrolyze broad-spectrum cephalosporins like Ceftazidime, Ceftriaxone, Cefepime and monobactams such as Aztreonam and Cephamycins. Twenty-nine different plasmid-mediated AmpC genes have been identified. The phenotypic diagnosis of AmpC positive microorganisms is difficult and clinical laboratories cannot detect these bacteria. In fact, resistance to β-lactam antibiotics in those bacteria that typically harbor co-existence of AmpC and ESBL enzymes are not distinguishable and known as β-lactamase producers.

Considering importance of β-lactamases in Klebsiella spp, the objectives of this study were to phenotypically detect ESBL, AmpC and MBL enzymes in Klebsiella spp isolated from paderaite patients admitted in The Children's Hospital Lahore. Afterwards, molecular characterization of ESBL, AmpC and MBL genes was done in these strains by PCR. Finally, co-existence of ESBL, AmpC and MBL enzymes and genes in Klebsiella spp was determined.

METHODS

Research Settings

This cross-sectional study was conducted in the Department of Microbiology, The Children's Hospital and Institute of Child Health, Lahore from September 2019 to February 2020. A total of 259 samples were included in this cohort under 15 years of age with clinical suspicion of sepsis.

Sample Collection

Clinical samples of blood and urine were collected from various wards of The Children's Hospital & Institute of Child Health, Lahore, Pakistan and were

sent for microbiological studies to the Microbiology Department of the hospital. The samples were collected consecutively from pediatric patients. The demographic and clinical data of screened positive Klebsiella spp was collected from Cardiology, Gastroenterology, Hematology/Oncology, Medical Emergency, Medical Units, Nephrology, Neurology, Urology, OPD and Surgery wards of the hospital. This data included information on the gender, age and presenting complaints of the patients. The samples were processed for microbiological examination using one or more suitable media such as Blood, MacConkey and CLED agar.

Products, Chemicals and Media Preparations

All solutions, reagents, media, antibiotic discs and molecular kits used in the present study were of analytical grade. All solutions were prepared using either distilled water (dH_2O) or double-distilled water (ddH_2O; Milli-Q; Millipore). All solutions, glassware and media were sterilized by autoclaving at 121°C for 15 min. Thirty-nine grams of blood agar base (Oxoid) were suspended in 1 liter of dH_2O and autoclaved to prepare blood agar. Thirty-eight grams of Mueller-Hinton agar (Oxoid) were suspended in 1 liter of distilled water and autoclaved to prepare Mueller-Hinton Agar. Fifty grams of MacConkey agar base (Oxoid) were suspended in 1 liter of dH_2O and autoclaved to prepare MacConkey Agar. Thirty-eight grams of Cysteine Lysine Electrolyte Deficient Medium (CLED) agar (Oxoid) was suspended in 1 liter of distilled water and autoclaved. Glycerol stocks were prepared in 1.5 ml cryovials using 15% glycerol (v/v/) in Brain Heart Infusion (BHI) broth. Tris EDTA (TE) buffer (1x) contained 10 mM Tris and 1 mM EDTA (pH 8.0). Tris-acetate-EDTA (TAE) buffer contained 0.04 M Tris, 5.7% (v/v) glacial acetic acid and 1 mM EDTA (pH 8.0). DNA gels generally contain 1% (w/v) agarose (Biorad) in 1x TAE buffer. Agarose gel contained 1:10,000 diluted SYBR Safe (Invitrogen) to permit DNA visualization under UV light. Recipes are shared in supplements.

Identification of Bacteria

The bacteria were identified using colony morphology, Gram's stain, biochemical tests, API (bioMerieux) 10S. For the identification of Klebsiella spp, large grey-white colonies on Blood agar, large pink (lactose fermenting) colonies from MacConkey agar and yellow mucoid (lactose fermenting) colonies from CLED agar were processed for further biochemical tests. A well-isolated colony from the culture plate was emulsified in a drop of sterile dH_2O on a slide to make a thin preparation. The smear was dried in air and heat-fixed by passing over the flame three times. The smear was then covered with crystal violet for 60 seconds and washed off with tap water. Then smear was covered with Lugol's iodine for 60 seconds and washed off with water. Then it was decolorized for few seconds with acetone-alcohol (1:1) and washed with water. The smear was counter-stained with safranin for 2 minutes and washed with water. The slides were air dried and examined using a 100X objective of microscope.

Biochemical Test

Various biochemical tests were done to test the suspected colonies of Klebsiella spp that showed negative motility, citrate positive, urease positive, and on TSI they gave alkaline slants (yellow) and alkaline butt (yellow) with gas production and no blackening. Recipes for the media are shown in supplements.

Analytical Profile Index

Analytical Profile index (API) 10S is a set of 11 biochemical reactions carried out in mini wells which contain substrate in them and interpreted using kits and databases. The wells containing dehydrated substrate are present on API 10S strip used to identify bacterial members of the Enterobacteriaceae family. The suspension of test sample was made by mixing a well isolated colony in saline water. Then by using sterile syringe, suspensions were inoculated into the wells of strip. One well-containing CIT substrate was filled completely while the remaining were half filled. Mineral oil was added in LDC, ODC, H2S and URE to provide anaerobic conditions for their reactions, covered according to the manufacturer's instructions (bioMerieux) and incubated over-night at 36°C. The results of all the biochemical reactions were noted and a 4-digit code generated was used to identify the bacterial species using API software or an identification manual.

Antimicrobial Sensitivity Test

The antimicrobial resistance and susceptibility pattern of Klebsiella spp was determined using Kirby-Bauer disc diffusion method. A bacterial suspension was made according to the McFarland 0.5 turbidity standard and an even lawn of bacteria made on the Muller-Hinton agar plate. Antibiotic discs were placed on two different agar plates using a disc dispenser (Oxoid) and plates were incubated overnight at 35±1°C. The antibiotic discs (Oxoid) used in the present study were Amikacin, Amoxicillin-clavulanate acid, Cefotaxime, Ceftriaxone, Ceftazidime, Cefoxitin, Cefuroxime, Cefepime, Ciprofloxacin, Gentamicin, Imipenem, Meropenem, Piperacillin-tazobactam, Tobramycin, Trimethoprim-sulfamethoxazole and Sulbactam. The zone sizes of these antibiotics were measured in mm and the Klebsiella spp isolates were interpreted as susceptible or resistant according to the CLSI manual (CLSI, 2009).

ESBL Screening and Confirmation

Extended spectrum β-lactamase (ESBL) screening was performed using antibiotic discs of ceftazidime or cefotaxime (Oxoid). Klebsiella spp strains resistant to any of these indicator drugs were considered as ESBL screen positive and were processed further for confirmatory tests. A double disc synergy test (DDST) was performed by placing a disc containing amoxicillin-clavulanate on an inoculated Mueller-Hinton agar plate at 20mm distance from the ceftazidime and cefotaxime. ESBL production was noted by the clavulanate mediated enhancement of the activity of ceftazidime or cefotaxime as a keyhole effect. The CLSI confirmatory test for ESBL detection was performed by placing cefotaxime and ceftazidime discs alone and in combination with cefotaxime-clavulanate and ceftazidime-clavulanate (Oxoid) on an inoculated Mueller-Hinton agar plate. The CLSI test using combined discs of cefotaxime-clavulanate or ceftazidime-clavulanate confirmed ESBL production in Klebsiella spp when the inhibition zone of any of the above-mentioned cephalosporin increased at least ≥5 mm in the presence of clavulanate (CLSI, 2009).

AmpC Screening and Confirmation

All the strains were screened for the AmpC β-lactamase production by the Kirby Bauer disk diffusion test. The isolates which showed reduced susceptibility to Cefoxitin were tested for confirmation by the modified three-dimensional tests. AmpC production was detected and confirmed by 3-aminophenyl Boronic acid (APB; Sigma Aldrich, India) disk potentiation test, 5µl of APB stock solution (240 mg APB in 3 ml of Dimethyl sulfoxide (DMSO)) was added to Cefotaxime (CTX), Ceftazidime CTZ, and Cefoxitin (FOX) disks. The final concentration of APB on each disk was 400µg. A ≥5mm increase in zone of CTX and/or CTZ and/or FOX disk alone and in combination with APB was considered AmpC production (CLSI, 2009).

MBL Screening and Confirmation

The metallo-β-lactamase production was screened by Kirby Bauer disk diffusion method. The isolates which showed reduced susceptibility to imipenem or meropenem were tested for confirmation. The metallo-β-lactamase production was detected by Imipenem–EDTA double disc synergy test. A 0.5M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA $2H_2O$ in 1,000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10µg imipenem disks (Becton Dickinson) were placed on the plate, and appropriate amounts of an EDTA solution were added to one of them to obtain the desired concentration. The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 h of incubation in air at 35°C. The organisms were MBL producers because increase in the inhibition zone of the Imipenem+ EDTA disk was ≥ 5 mm.

Molecular Studies

The analyses of ESBL genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M} group), MBL gene (NDM-1) and AmpC genes (CIT and EBC) were performed. For this purpose, ESBL, MBL or AmpC producing Klebsiella spp strains were re-energized by sub-culturing isolates on MacConkey agar after overnight incubation at 37±1°C. The DNA templates were extracted by taking three well isolated colonies of Klebsiella spp grown overnight on MacConkey agar and suspending the colonies in 200µl of TE buffer. The turbidity of suspension was adjusted according to the 0.5 McFarland's turbidity standard. The suspension was boiled in a 1.5 ml microcentrifuge tube for 10 minutes. The lysed bacterial cells were centrifuged at 14000 x g for 5 minutes and the clear supernatant was transferred to a new microcentrifuge tube and stored at -20°C until required.

Amplification of Genes

The amplification of genes was performed by polymerase chain reaction (PCR) in a Thermal Cycler. Each PCR reaction consisted of 10µl Dream Taq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 7µl of nuclease-free water, 1µl of each of the 10 primers solutions and 1µl DNA lysate. Running conditions were 1 cycle of denaturation at 94ºC for 15 min followed by 25 cycles of denaturation at 94ºC for 30 sec, annealing at 58ºC for 90 sec, elongation at 72ºC for 60 sec and a final cycle of elongation at 72ºC for 10 min. Amplified PCR products were analyzed by agarose gel electrophoresis and when required mixed with 6x Gel Loading Buffer (New England Bio Labs) prior to loading. A 100 bp ladder was used to determine the size and approximate DNA concentration (New England Bio Labs). Electrophoresis was performed at 96V for 40-45 min. DNA products were visualized on a G: Box UV Transilluminator (Syngene).

Statistical analysis:

Data collected in proforma was entered and statistical analysis and calculations were done using the Microsoft Excel 2016 and IBM SPSS statistics 25.0 Software. Categorical variables were expressed in frequencies and percentages. Graphs were used to display the data.

RESULTS

Patients Cohort Details

Patients described in this study were admitted to the Children's Hospital and Institute of Child Health Lahore, Pakistan during Sep 2019 to Feb 2020. All patients were aged between one day and 15 years. Over 6 months study period, 3516 patients presented symptoms suspected of bacterial infections, and microbiological testing was ordered by the clinicians for them. Out of 3516 patients sampled, 259 were tested positive for infection against Klebsiella spp, identified using various culture media and biochemical tests. During the study period, 259 isolates of Klebsiella spp were isolated from various clinical samples 64.5% were male and 35.5% were female patients (Table 1). The patients were recruited from various wards of the hospital as shown in Table 2. Frequency of positive and negative cultures among the sample collected is reflected in Table 3.

Phenotypic Detection of Enzymes

ESBL production in Klebsiella spp isolates was initially screened and confirmed based on resistance to ceftazidime or cefotaxime and Double disk synergy test. The number of ESBL positive and negative isolates were 53 (20.5%) and 206 (79.5%), respectively. These isolates were screened for MBL production and confirmed by Imipenem – EDTA double disc synergy test. The number of MBL positive and negative isolates were 64 (24.7%) and 195 (75.3%), respectively. These isolates were also screened and confirmed for AmpC production by 3-aminophenyl Boronic acid disk potentiation test. The number of AmpC positive and negative isolates were 86 (33.2%) and 173 (66.8%), respectively (Table 4). The isolates showed positivity to screening test were processed for further molecular analyses.

Genetic Expressions in Klebsiella spp isolates

The bla genes (SHV, TEM and CTX-M) which encode for the ESBL production were detected using PCR. Amplification of the bla genes was done with specific primers and amplified genes were detected with agarose gel electrophoresis. The results of the PCR amplification of the genes showed the presence of SHV in 22 (8.5%) isolates, CTX-M in 14 (5.4%) isolates and TEM in 2 (0.8%). NDM-1 gene for MBL production was detected using the same method with specific primer. The results of amplification showed presence of genes in 59 (22.8%) isolates. CIT and EBC genes were also detected for AmpC production and amplification showed 30 (11.6%) isolates that carry CIT genes and 12 (4.6%) were having EBC gene in them. Two isolates co-produced all three enzymes followed by nine that co-produced MBL and ESBL, four isolates co-produced AmpC and MBL and four co-produced ESBL and AmpC. Frequency of various genes in β lactamases are shown in Table 5.

Table 1: Age and Gender Distribution of Septic Patients

Age Groups	**Frequency**	**Percentage (%)**
0-5 years	91	35.1
6-10 years	114	44.1
Gender	**Frequency**	**Percentage (%)**
Male	167	64.5
Female	92	35.5
Total	**259**	**100**

Table 2: Distribution of Samples Among Various Wards

Wards	**Frequency**	**Percentage (%)**
Nephrology	53	20.5
Emergency wards	43	16.6
Medical wards	39	15.1
OPD	30	11.6
Neurology	27	10.4
Urology	26	10.0
H/O	14	5.4
Cardiology	11	4.2
Surgery wards	10	3.9
Gastrology	6	2.3
Total	**259**	**100**

Table 3: Frequency of Positive and Negative Cultures Among the Sample Collected

Specimen	Positive cultures	Negative cultures
Blood	132	2802
Urine	127	714
Total	**259**	**3516**

Table 4: Phenotypic detection of Enzymes from Klebsiella spp isolates

Enzymes	Present	Absent
ESBL	53	206
MBL	64	195
AmpC	86	173

Table 5: Frequency of Various Genes in β-lactamases

Genes	Frequency	Percentage (%)
ESBL- SHV	22	8.5
ESBL- CTX-M	14	5.4
ESBL- TEM	2	0.8
MBL- NDM-1	59	22.8
AmpC- CIT	30	11.6
AmpC- EBC	12	4.6

DISCUSSION

The isolation and identification of bacterial pathogens is important for diagnostic, therapeutic and infection control purposes. Most of the bacterial infections are caused by Gram negative bacteria including Klebsiella spp. Klebsiella spp is an important pathogen isolated from both community and hospital-acquired infections. Klebsiella spp remained a common microorganism responsible for various infections among hospitalized patients [13].

The present study was conducted in Microbiology Department of the Children's Hospital and Institute of child Health Care, Lahore during a period of six months. Main objectives of this study were to phenotypically detect various β-lactamases including ESBL, MBL, and AmpC, alongside molecular characterization of the genes and to determine co-existence of enzymes

In this study, 259 isolates of Klebsiella spp were collected from different clinical specimen including Blood (50.9%) and Urine (40.1) and were screened and confirmed for ESBL, MBL and AmpC production. It was observed that 206/259 (78.3%) isolates produced at least one type of β lactamase. These results are in accordance with the study conducted in Babol University of Medical Sciences, Iran to detect phenotypically ESBL, MBL, and AmpC enzymes that showed 38/59 (64.4%) isolates of Klebsiella spp produced at least one type of β-lactamase [14]. The frequency of bacteria remained different from various samples in different studies.

In the present study, phenotypical detection showed the frequency of ESBL 53 (20.4%), MBL 86 (33.2%) and AmpC 64 (24.7%) among all positive cultures. Similar study conducted by Jena et al., on the prevalence of ESBL, MBL, and AmpC β-lactamases producing multidrug resistance (MDR) gram negative bacteria in tertiary care hospital showed ESBL production in 118 (31.54%), MBL in 115(19.29%), and AmpC in 306 (51.34%) along with the co-production of ESBL/MBL/AmpC β-lactamases was observed in 105 (20.87%) strains [15].

The frequency of patients infected with Klebsiella spp was high in nephrology ward (20.5%) followed by Emergency wards (16.6%), Medical wards (15.1%), OPD (11.6%), Neurology (10.4%), Urology (10.0%), H/O (5.4%), Cardiology (4.2%), Surgery wards (3.9), and Gastrology (2.3%). Khan et al. studied the outbreaks of Klebsiella infections and concluded that majority of these infections occurred among the children from neonatology ward [16]. Another study from Brazil also reported the outbreaks of ESBL-producing Klebsiella spp. from neonatal nursery unit. It was found that 22.5% of the neonates admitted during the three-month period were colonized with Klebsiella spp [17]. Other studies reported the incidence of Klebsiella infections from intensive care

units, neurosurgery, pulmonary internal medicine, general medicine and clinic of anesthesiology [18].

The total number of ESBL producing Klebsiella spp initially identified based on screening and confirmatory test were 53 (20.4%) out of 259 isolates. A significant increase in ESBL Klebsiella spp was noted during a study conducted in Aga Khan University Hospital Karachi, Pakistan [16]. Riaz et al. reported 26.1% ESBL Klebsiella spp isolated from various specimens in Lahore, Pakistan [19]. A very high number of ESBL (70.0%) were isolated from Pakistan Institute of Medical Sciences [20]. Another study reported 97.67% of ESBL Klebsiella spp collected from various hospitals in Iraq [21]. The frequency of ESBL Klebsiella spp differs all over the globe depending upon the infection control measures. The frequency of ESBL Klebsiella spp in the present study was relatively low.

In the current study 24.7% Klebsiella spp. were AmpC positive. A conducted study on 100 isolates of Klebsiella spp in which 32 (32%) were AmpC β-lactamase producers. Nahid Hoseini et al conducted a study at Hamadan University of Medical Sciences, Iran and found 48.5% Klebsiella spp were AmpC producers [21]. Hemalatha et al (2007) in their work at Sri Ramachandra Medical College and Research Institute Chennai, in India found 47.3% isolates of AmpC β-lactamase producers. This variation may be due to different cleaning and infection control practices.

In the specimens of blood and urine, 33.2% were resistant to both Meropenem and Imipenem and were confirmed that they produced MBL by Imipenem–EDTA double disk synergy test. Aljanaby et al conducted their study in Iraq and concluded that 9.30% isolates were MBL producers [21]. Similarly, Jena et al at Tertiary care Hospital showed that 19.29% of isolates were MBL producers. MBL Production varies in different studies [15].

Various genes in β-lactamases were detected based on molecular analysis that showed three genes of ESBL i-e SHV (8.5%), CTX-M (5.4%) and TEM (0.8%). The results were comparable with previous studies. Aljanaby et al conducted his study in Iraq and showed high prevalence of SHV gene (86.04%) for ESBL production. Mendonca et al conducted their study in which 11 isolates have SHV gene and 9 isolates of Klebsiella spp had TEM gene [22]. Joel et al showed in their study that among 78.3% ESBL producers, SHV genes were seen in 83.3% whereas CTX-M and TEM gene were 56% [23]. Chin Fu et al also showed in their study that SHV gene was most dominant (57.6%) among Klebsiella spp. In the current study frequency of NDM-1 genes in MBL producers was (22.8%) [24]. Occurrence of genes in AmpC producing strains of Klebsiella spp was different in a study conducted by Nahid Hoseini et al in which 35.2% had CIT gene and 61.7% had EBC gene [25]. In the present study co-existence of enzymes was checked and observed that 9 (3.5%) isolates co-produced MBL+ ESBL followed by 4 (1.5%) that co-produced AmpC+ ESBL and 4 (1.5%) co-produced AmpC+MBL. All three enzymes were present in 2 (0.8%) Klebsiella isolates. Jena et al conducted a study to determine co-existence of ESBL, MBL and AmpC among all gram-negative isolates and observed co-production of 20.87% [15].

To conclude, antibiotic resistance is posing serious implications in developing countries like Pakistan. Bacteria develop mechanisms to resist the effect imposed by the antibiotics. This study focused on demographic, clinical, laboratory and molecular aspects of the infected patients with Klebsiella spp, presence of various β-lactamases and co-existence of enzymes. A large number of patients were found to have serious bacterial infections with significant number of ESBL, MBL and AmpC-production along with co-existence. Multi drug resistant Klebsiella spp is a serious public concern. Hence, early detection and reporting of β lactamases is important for the successful treatment of patients.

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Ethics Approval: Study was approved from the relevant ethical board of the institute.
Competing Interests: There are no competing interests among the authors.
Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.