

Antibiotic Resistance of Clinical *Klebsiella* Isolates from Tertiary Care Hospital

Ramya Sivaramakrishnan, K.V. Leela, R. Sujith* , Shweta Naik and Sneha Priya

Department of Microbiology, SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, SRM Nagar, Kattankulathur, Chennai District, Tamil Nadu, India.

Abstract

Klebsiella is a pathogen that causes a significantly high number of community-acquired and hospital-acquired infections, with infections being one of the leading causes of death in ICU patients worldwide due to increasing antibiotic-resistance and a lack of therapeutic options. A total of 230 *Klebsiella* spp. were collected from various clinical samples. After initial identification, the drug-resistant strain was subjected to standard Clinical Laboratory and Standards Institute methods such as Kirby–Bauer disc diffusion. All isolates were screened and confirmed for ESBL/AmpC β -lactamase/carbapenemase production. The isolated *Klebsiella* spp. were found to be *K. pneumonia* (89%), *K. oxytoca* (6.5%), and *K. aerogenes* (4.5%). Among the 230 isolates, 80 (34.7%) isolates were found to be ESBL producers via screening; of these, 53 (23.5%) were verified by a confirmatory test. Moreover, 115 isolates (50%) were screened as AmpC producers; of these, 23 isolates (10%) were verified by a confirmatory test. Carbapenemase producers accounted for 69 (30%) isolates, identified by screening; 25 (10.86%) were verified by a confirmatory test. ESBL producers accounted for the majority of *Klebsiella* spp. isolates, followed by carbapenem and AmpC producing strains.

Keywords: *Klebsiella*, Antibiotic Resistance, ESBL, Amp C β -lactamase, Carbapenemase

*Correspondence: sujithr@srmist.edu.in

Citation: Sivaramakrishnan R, Leela KV, Sujith R, Naik S, Priya S. Antibiotic Resistance of Clinical *Klebsiella* Isolates from Tertiary Care Hospital. J Pure Appl Microbiol. 2022;16(4):2669-2678. doi: 10.22207/JPAM.16.4.36

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INTRODUCTION

Klebsiella is a bacterial genus in the *Enterobacteriaceae* family, which is gram negative, non-motile, non-spore-forming bacilli that normally reside in the mammalian gastrointestinal tract and infect the host opportunistically.¹ These bacteria often cause life-threatening infections such as septicemia, urinary tract infections, pneumonia, and liver abscess in immuno-compromised patients.² *Klebsiella pneumoniae* is the most prevalent nosocomial and opportunistic pathogen that causes infections in humans among *Klebsiella* spp.³

The emergence of antimicrobial resistance in *Klebsiella* spp. is becoming a global threat owing to increasingly ineffective treatment options. In empirical treatment protocols, resistant screening plays an important role. Bacteria produce various enzymes that promote drug resistance. Extended spectrum beta lactamases (ESBLs) are enzymes that hydrolyze most penicillins and cephalosporins, including oxyimino- β -lactam compounds (i.e., cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but not cephamycins nor carbapenems. Most ESBLs belong to the Ambler class A of β -lactamases and are inhibited by β -lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) and bi-diazabicyclooctanes (avibactam).⁴ These enzymes are primarily responsible for resistance in *Klebsiella* spp. In *Klebsiella pneumoniae*, the primary factor influencing high ESBL production is the usage of third-generation cephalosporins. Other risk factors include long-term hospitalization in the intensive care unit (ICU), catheterization, mechanical ventilation, preterm birth, and low weight at birth.⁵

AmpC-beta lactamases are Ambler class C β -lactamases that hydrolyze penicillins, cephalosporins (including third-generation but generally not the fourth-generation compounds) and monobactams.⁶ In general, AmpC-type enzymes are poorly inhibited by classical ESBL inhibitors, as clavulanic acid mediates resistance to antibiotics such as cefotetan and ceftiofur and is produced by some strains of *Klebsiella* spp.⁷⁻⁹

Carbapenemases are β -lactamases that hydrolyze penicillins, in most cases cephalosporins and, to various degrees, carbapenems and

monobactams. The latter are not hydrolyzed by metallo- β -lactamases. The carbapenem resistance of *K. pneumoniae* is a major reason for the strain's high morbidity and mortality.¹⁰ Hence, studying the characteristics that promote resistance in this strain will promote the effective use of antibiotics in treatment. Moreover, studying antibiotic-resistant *K. pneumoniae* elucidates the importance of screening and confirmatory methods for research and treatment.¹¹

The screening test alone is insufficient for detecting antibiotic resistance. Hence, there is a need for reliable phenotypic confirmatory testing to identify resistant *Klebsiella* spp. in clinical samples. The present study emphasized the importance of confirmatory tests compared to screening tests and provided foundational data for the appropriate use of antibiotics.

MATERIALS AND METHODS

Methods

Identification

Clinical samples were obtained in an aseptic manner and processed using standard microbiological techniques. Ethical approval was obtained from the Institutional Ethics Committee of SRM Medical College Hospital and Research Center (1860(A)/IEC/2019). Conventional culture techniques were used to isolate the *Klebsiella* spp. After obtaining the sample, direct gram stain was performed, and samples were cultured on blood agar, MacConkey agar, and chocolate agar. All suspected isolates are identified by Gram stain and hanging drop method. *Klebsiella* spp. were speciated using the following biochemical tests: indole, methyl red, Voges-Proskauer, triple sugar iron, urease, citrate, OF dextrose, nitrate reduction, lysine, ornithine, and arginine.^{12,13} Antibiotic susceptibility testing (AST) was carried out according to the Clinical and Laboratory Standards Institute (CLSI) using Kirby-Bauer disc diffusion method in Muller-Hinton agar.¹⁴

AST Procedure

AST was conducted according to drugs listed in the CLSI guidelines for *Klebsiella* spp. Testing was carried out using Muller-Hinton Agar (MHA) with the Kirby-Bauer disc diffusion method. The following antibiotic discs were used: ampicillin

(10 µg), amoxicillin–clavulanate (20 µg/ 10 µg), ceftazidime (30 µg) ceftazidime with clavulanic acid (30 µg/20 µg), cefepime (30 µg), ceftriaxone (30 µg), ceftizoxime (30 µg), ertapenem (10 µg), meropenem (10 µg), piperacillin tazobactam (100 µg/10 µg), amikacin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), ceftaxitin (30 µg), ceftazolin (30 µg), gentamycin (10 µg), imipenem (10 mg), tetracycline (30 µg), tigecycline (15 µg), and nitrofurantoin (300 µg) (purchased from Himedia, Mumbai, India). The zone size was interpreted after 18–24 h of incubation according to CLSI guidelines.

Screening Tests for ESBL, AmpC, and Carbapenemase Producers

ESBL screening test was performed according to CLSI guidelines by a disk diffusion method in which the isolate was streaked as a lawn culture over MHA and antimicrobial concentrations of cefotaxime (30 µg) and ceftazidime (30 µg) were used. If the zone size was <22 mm and <27 mm after 18–24 h of incubation, the isolate was considered positive for ESBL production.

AmpC screening test was performed according to EUCAST guidelines¹⁵ by a disk diffusion method in which the isolate was streaked as a lawn culture over MHA and an antimicrobial concentration of ceftaxitin (30 µg) was used. If the zone size was ≤14 mm after 18–24 h of incubation, the isolate was considered positive for AmpC production.

Carbapenemase screening test was performed based on CLSI guidelines using a disk diffusion method in which the isolate was streaked as a lawn culture over MHA and an antimicrobial concentration of ertapenem (10 µg) was used. If the zone size was ≤ 19 mm after 18–24 h of incubation, the isolate was considered positive for carbapenemase production.

Confirmatory Tests for ESBL, AmpC, and Carbapenemase Production

Combined testing for ESBL detection was carried out based on CLSI guidelines using a disk diffusion method in which the isolate was streaked as a lawn culture over Muller–Hinton agar and two antibiotic discs were used: ceftazidime (30 µg) and ceftazidime with clavulanic acid (CAC) (30 µg/20 µg). The CAC disc was placed in the center of the

MHA plate, the and ceftazidime disc was placed approximately 1.5 cm away from the CAC disc. If the zone size difference for each exhibited a ≥5 mm increase in inhibition zone, the sample was confirmed for ESBL production.

Double Disc Synergy Test

The double disc synergy test relied on the ability of cloxacillin to inhibit AmpC enzyme function. This test was conducted based on EUCAST guidelines. Ceftaxitin (30 µg) and cloxacillin (200 µg) discs were used (Himedia, Mumbai). The strains were streaked as a lawn culture on MHA with 0.5 McFarland and incubated for 18–24 h at 37°C. A zone of inhibition ≥4 mm between the ceftaxitin–cloxacillin and ceftaxitin zones confirmed AmpC production.

Modified Hodge Test (MHT)

MHT

To verify carbapenemase enzyme production, the MHT was conducted according to CLSI guidelines. A carbapenem-susceptible strain of *E. coli* ATCC 25922 is known to grow toward the carbapenem from the test isolate, which also generates the enzyme.

Testing Procedure

An overnight culture of *E. coli* ATCC 25922 (indicator) yielded a 0.5 McFarland standard suspension, which was diluted 1:10 in saline or broth. The suspension was inoculated onto the MHA plate using lawn culture and left to dry for 3 to 10 min. In the center, a 10 g ertapenem disc (Himedia) was placed. The test isolate and positive and negative controls were streaked from the edge of the disc to the plate's periphery and incubated at 37°C for 24 h. Streaks were 20 to 25 mm in length. If the test strain showed growth near the inhibition zone, carbapenemase production was assumed. If the test strain did not grow near the inhibition zone, carbapenemase was not produced.^{16,17}

RESULTS

Isolate Identification

In this study, 230 isolates of *Klebsiella* were collected and subjected to identification by microbiological culture, and biochemical

tests and AST were performed for verification. Microbiological culturing was performed using MacConkey agar, blood agar, and biochemical solutions to validate *Klebsiella* spp. identification. Of the 230 isolates, the following strains were identified: *K. pneumonia* (89%), *K. oxytoca* (6.5%), and *K. aerogenes* (4.5%) (Figure 1). Nearly 65% of the *Klebsiella* spp. were isolated from male patients, and 35% of *Klebsiella* spp. were isolated from female patients. Of the 230 isolates, 36.5% were obtained from patients in the age group 40–60 years of age, followed by 35.6% from patients in the age group of 20–40 years, 30.19% in the age group of 60–80 years, followed by (2.6%) followed by (6%) followed by 1-20 years (Figure 2).

Sample Distribution

The sources of infection in the collected *Klebsiella* samples were found to be urine (30.43%), sputum (20.43%), pus (16.08%), tracheal aspirate (6.95%), wound swab (6.95%), vaginal blood swab (4.34%), ear swab (3.47%) and body fluids (3.47%), (Figure 3) respectively. The spectrum of disease distribution of studied isolates was as follows: urinary tract infections, chronic illness, wound infections, diabetic foot ulcer, Respiratory tract infection and septicaemia (Figure 4) The ward distribution of studied isolates was as follows: ICU (26.08%), general surgery (17.39%), general medicine (17.39%), labour ward (10.86), pulmonary ward (10%), orthopaedic ward

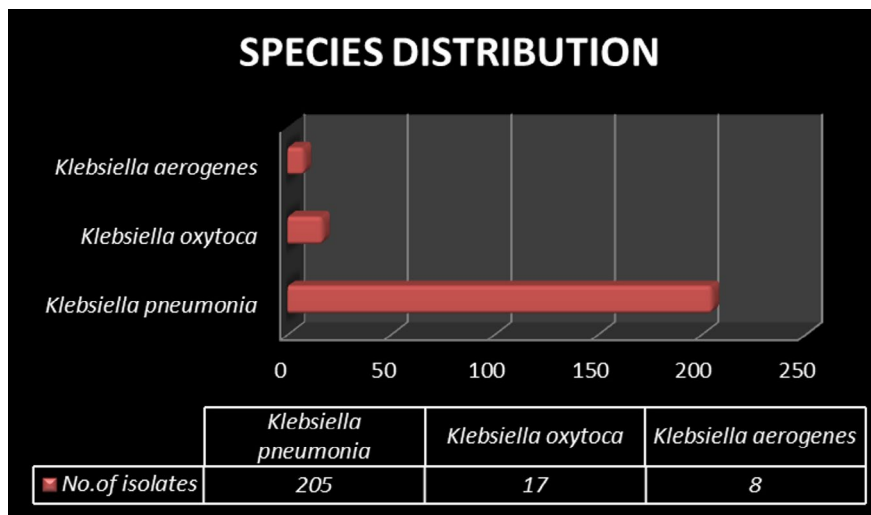


Figure 1. Among 230, isolates 89% of isolates belongs to *Klebsiella pneumoniae*

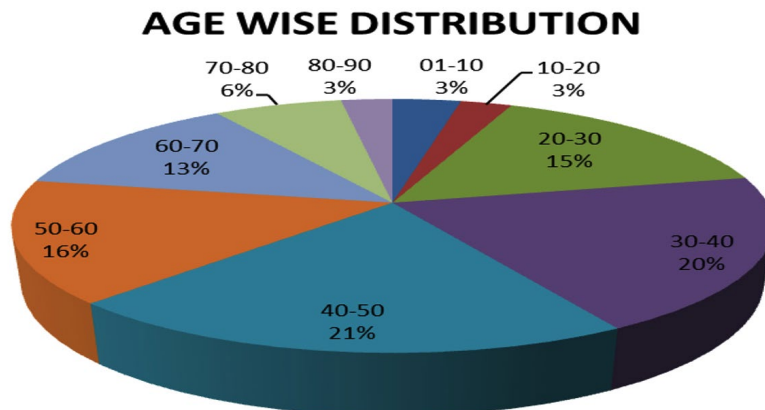


Figure 2. Patients in age group of 40-60 years of age are highly affected by *Klebsiella* infections

(20%), urology ward (7.82%), COVID-19 ward (2.17%), and burn ward (0.43%) (Figure 5).

AST Results

AST and ESBL, AmpC β -lactamase, and carbapenemase production analyses were carried out according to CLSI guidelines. The ESBL producers yielded zone size differences of ≥ 5 mm between ceftazidime and ceftazidime with clavulanic acid. Cefoxitin resistance was considered to indicate that the isolate was a AmpC producer. Imipenem, meropenem, and ertapenem resistance indicated that the isolate was a carbapenemase producer. The distribution of resistances among all isolates were determined

as follows: amoxicillin-clavulanic acid (20/10 μg) (50.86%), cefazolin (30 μg) (49.56%), ceftriaxone (30 μg) (47.39%), cefotaxime (30 μg) (46.52%), cefepime (30 μg) (46%), nitrofurantoin (300 μg) (44%), cefoxitin (30 μg) (43.47%), ceftazidime (30 μg) (37.82%), piperacillin-tazobactam (100/10 μg) (31.73%), ciprofloxacin (5 μg) (31.30%), ertapenem (10 μg) (30%), amikacin (30 μg) (30%), gentamicin (10 μg) (29.13%), meropenem (10 μg) (25.21%), ofloxacin (5 μg) (24.78%), imipenem (10 μg) (20.43%), chloramphenicol (30 μg) (15.21%), and tetracycline (10 μg) (12.06%) (Figure 9). *Klebsiella* spp. strains were the most resistant to amoxicillin and clavulanic acid.

SAMPLE WISE DISTRIBUTION

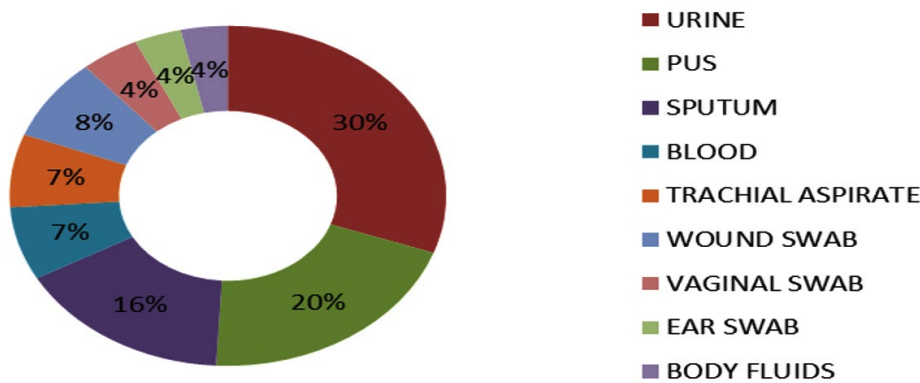


Figure 3. Highest number of *Klebsiella* spp. are isolated from urine sample

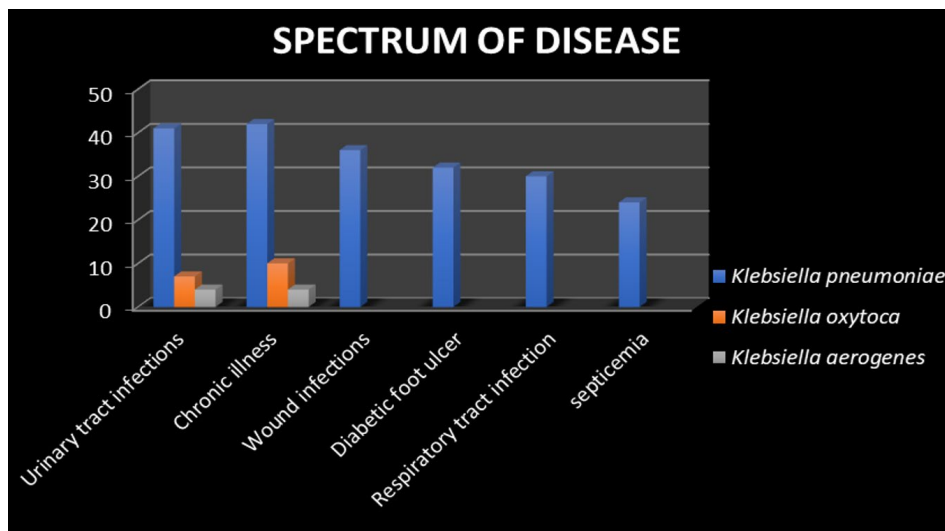


Figure 4. Majority of the *Klebsiella* spp. are isolated from urinary tract infections

ESBL, AmpC, and Carbapenemase Producer Confirmatory Test Results

- a. **Combined Disc Diffusion Test for ESBL Production** (Figure 6)
Of the 230 isolates, 80 (34.7%) isolates were found to be ESBL producers by the screening test; of these, 53 (23.5%) were verified as ESBL producers by the confirmatory test.
- b. **Double Disc Synergy Test for AmpC Production** (Figure 7)
Of the 230 isolates, 115 isolates (50%) were found to be AmpC producers by the screening test; of these, 23 isolates (10%) were verified as AmpC producers by the confirmatory test.
- c. **MHT for Carbapenemase Production** (Figure 8)

to be carbapenemase producers by the screening test; of these, 25 (10.86%) were verified as carbapenemase producers by the confirmatory test.

DISCUSSION

Among 230 *Klebsiella* spp. isolates, we identified the following strains: *K. pneumonia* (89%), *K. oxytoca* (6.5%), and *K. aerogenes* (4.5%). Similar results were suggested by Sandeep Vasikar et al.¹⁸ Male predominance is more nearly 65% of the *Klebsiella* spp. was isolated from male patients and 35% of *Klebsiella* spp. is isolated from female

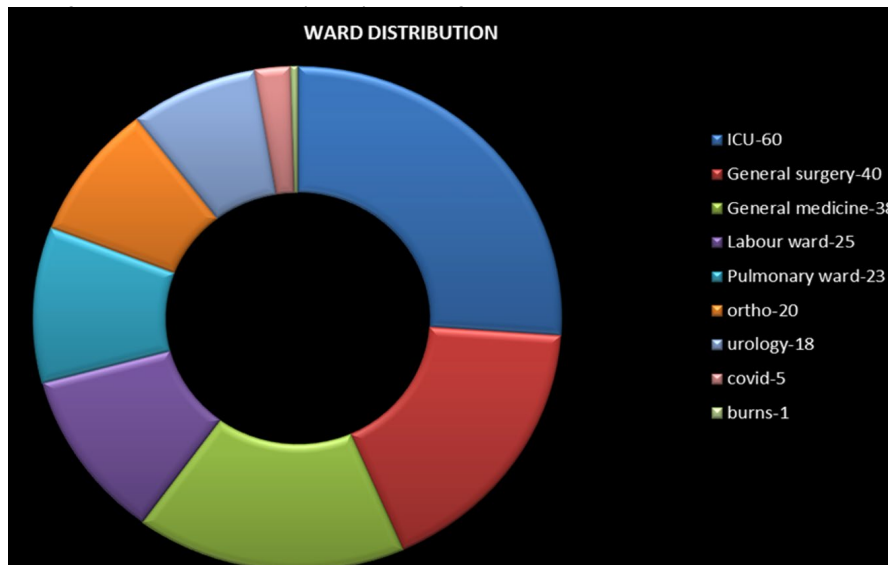


Figure 5. Nearly 60% of the *Klebsiella* spp. are isolated from ICU

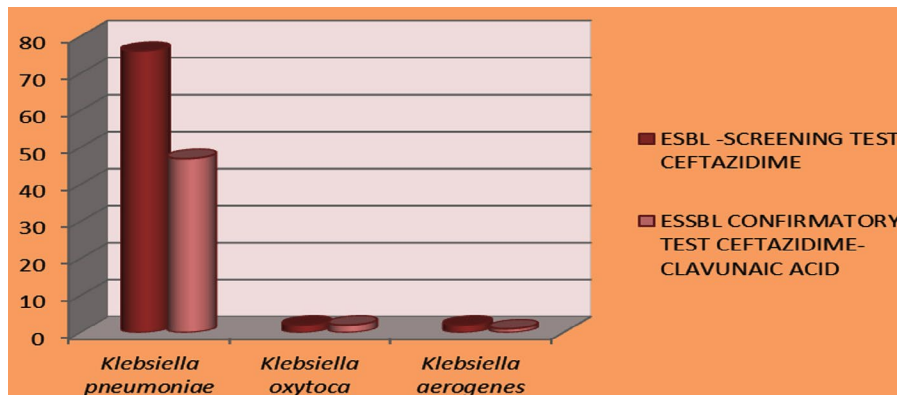


Figure 6. Among 230 isolates,80 (34.7%)isolates were found to be ESBL producer by screening test out of which 53 (23.5%) were ESBLproducer by confirmatory test

patients. Men are more commonly affected by *Klebsiella* infections than women due to the increased prevalence of alcoholism and smoking among men. This distribution is similar to that identified by Sunilkumarbiradar et al.¹⁹

In this study, isolate samples were predominantly obtained from patients in the age groups of 40–60 years (36.5%) and 20–40 years (35.6%). The next-most prevalent age group was 60–80 years (30.19%) followed by (2.6%) followed by (6%) followed by 1–20 years, which correlates with data reported by Virawan et al.²⁰ Infection is considered to be more common around 40–60 years of age because the immune declines as

individuals age and are associated with various co-morbidities. The highest number of *Klebsiella* spp. samples were isolated from urine followed by sputum, which reflects the findings reported by Hamida et al.²¹

Klebsiella spp. infections are the second-most common cause of urinary tract infection, which may be due to expression of type 1 fimbriae present in the urinary tract. Nearly one third (26.08%) of *Klebsiella* spp. samples are isolated from ICU patients because ICU patients are a tremendously vulnerable group and highly prone to infection, as reported by Aliyu Aminu et al.²² Carbapenem-producing *Enterobacteriaceae* pose

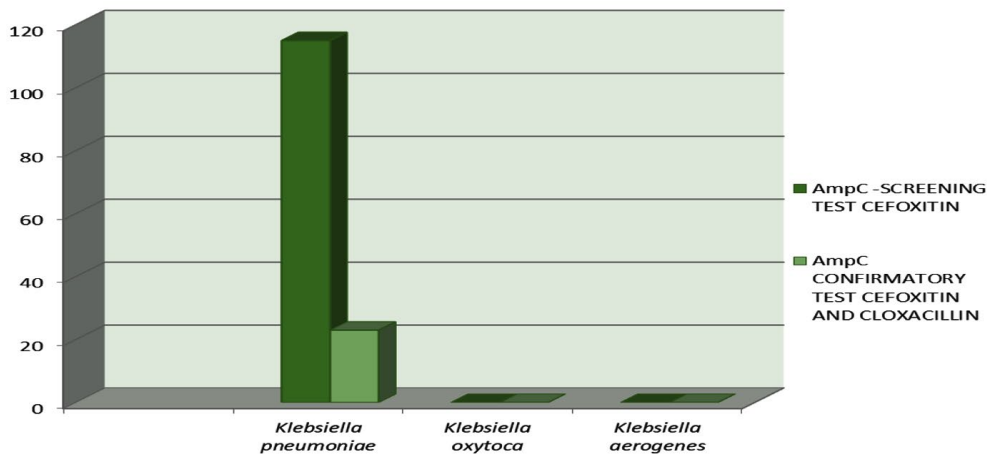


Figure 7. 115 isolates (50%) were found to be AmpC producer by screening test out of which 23 isolates (10%) was only positive in confirmatory test

CARBAPENEMASE TEST

■ Carbapenemase -SCREENING TEST ■ Carbapenemase CONFIRMATORY TEST

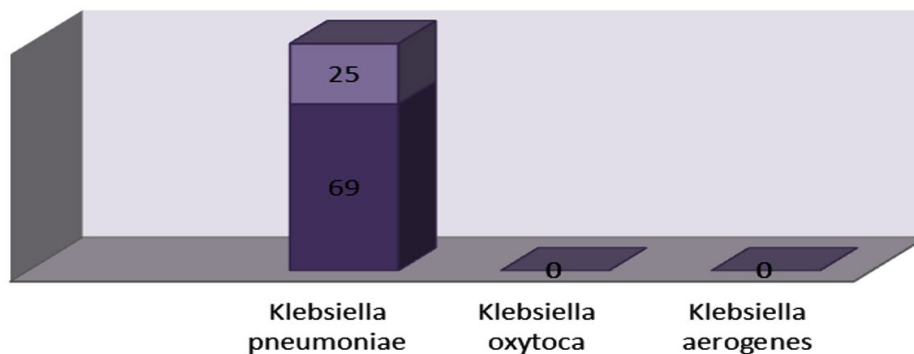


Figure 8. On analysis for Carbapenemase producer 69(30%) was isolated by screening test out of which 25(10.86%) isolates in confirmatory test were recorded

a significant threat to patients in the ICU, and *Klebsiella* spp. are the most common bacterial species in this group, causing life threatening infections. *Klebsiella* spp. were primarily resistant to amoxicillin and clavulanic acid, followed by cefazolin and ceftriaxone. The lowest resistance was to tetracycline, followed by chloramphenicol and imipenem. Using similar methods as those employed in this study, Mwangi Joseph Kibuchi et al.²³ reported that the highest drug sensitivities among *Klebsiella* spp. included that to meropenem, followed by amikacin and chloramphenicol. These discrepancies were expected because antibiotic susceptibility patterns change according to geographical location.

Among the 230 isolates, 34.7% were found to be ESBL producers by the screening method, and 23.5% were verified for ESBL production by the confirmatory method. The lower number of confirmed producers implies the importance of employing confirmatory tests to reduce false positive reporting, as this can lead to the increased unnecessary administration of antibiotics, which is known to promote drug resistance. Detecting ESBL producers among *Klebsiella* spp. is crucial as resistant strains are typically resistant to third-generation cephalosporins and aztreonam. Treatment failure occurs when the appropriate

antibiotic is not used. Moreover, ESBL producers often contain plasmids that carry genes encoding co-resistance to other antibiotics. Iran Hadi Mehrgan et al.²⁴ reported that ESBL producers exhibit around 70% now higher antibiotics like carbapenems are used as the drug against them.

Among the 230 isolates, 50% were found to be AmpC producers by the screening test, and 10% were verified as AmpC producers by the confirmatory test. These findings were similar to those reported by Donati et al.²⁵ This difference in initial and final identification further supports the necessity of confirmatory testing.

Nearly 30% of all isolates were determined to be carbapenemase producers by the screening test, and 10.86% were verified by the confirmatory test. The level of carbapenemase producers among our isolate samples was relatively low when compared with the findings of Shao et al.²⁶ who reported 23.3% of evaluated *Klebsiella* samples as carbapenemase producers. Carbapenems are widely administered to various treat-life threatening infections, and the development of resistance to this drug class will increase mortality, hospitalization length, and healthcare costs. Hence, the proper detection and administration of this antibiotic is critical for limiting the development of carbapenem resistance in major bacterial species.

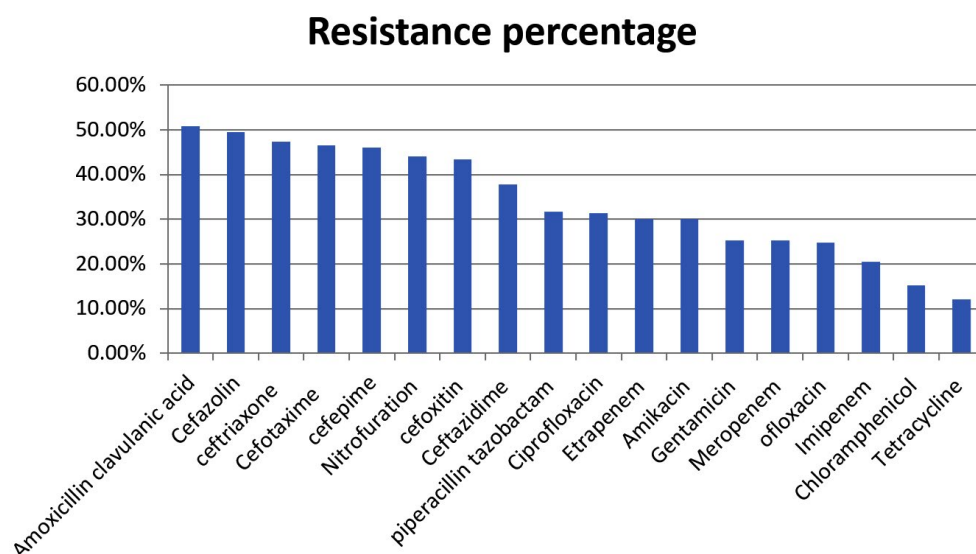


Figure 9. Results shows the lowest resistance rates to tetracycline drug in all isolates. So it could be considered to be more effective drug

CONCLUSION

The widespread and non-specific use of antibiotics results in an increasing number of multidrug-resistant *Klebsiella* spp. This study elucidated the enzymes responsible for drug resistance in *Klebsiella* spp. such as ESBL, AmpC β lactamase, and carbapenemases. Among 230 *Klebsiella* spp. isolates evaluated in this study, ESBL producers were most encountered, followed by carbapenemase and AmpC producers. Infection control practices along with antibiotic control policies are necessary for controlling increasing resistance trends. Moreover, employing confirmatory tests such as combined disc diffusion (ESBL detection), double disk synergy (AmpC detection), and Modified Hodge (Carbapenemase detection) tests in addition to screening tests is a reliable method for identifying drug resistance and can be carried out easily as a routine microbiology technique. Implementing confirmatory tests will promote the identification of antibiotic resistance to inform the careful and specific use of antibiotics in treating high-risk infections.

ACKNOWLEDGMENTS

The authors would like to thank SRM Medical College Hospital and Research Centre, Kattankulathur, Kanchipuram district, India for providing all the equipments and facilities for performing this research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, SRM Medical College Hospital and Research Centre, Tamilnadu, India (1860(A)IEC/2019).

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