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Evaluation of drug resistance in clinical isolates of klebsiella species

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Abstract:

Clinically significant, consecutive, non-duplicate isolates were included in the study. The significance of the isolate was based on two or more of the following criteria-clinical history, presence of organism in Gram stain, presence of intracellular forms of the organism and pure growth in cuture with a significant colony count wherever applicable. A total of 200 clinically significant, consecutive, non-duplicate isolates of Klebsiella spp. were included in this study. The isolates were from various clinical specimens sent to the Institute of Microbiology for bacteriological culture, biochemical identification and antibiotic susceptibility testing. Isolates included in this study were obtained from blood, sputum, endotracheal aspirate, bronchial wash, pleural fluid, ascitic fluid, peritoneal dialysis fluid, cerebrospinal fluid, urine and wound swabs.

Keywords: Klebsiella, isolation, culture specimen, growth of organism.

Introduction: Antimicrobial resistance (AMR) within a wide range of infectious agents is a public health threat of broad concern to countries. 1 Increasingly, governments around the world are beginning to pay attention to a problem so serious, that it threatens the achievements of modern medicine. AMR is a complex global public health challenge, and no single or simple strategy will suffice to fully contain the emergence and spread of infectious organisms that become resistant to the available antimicrobial drugs. The development of AMR is a natural phenomenon in microorganisms and is accelerated by the selective pressure exerted by use and misuse of antimicrobial agents in humans and animals. 2 The current lack of new antimicrobials on the horizon to replace those that become ineffective brings added urgency to the need to protect the efficacy of existing drugs. Hospitals, and particularly intensive care units, are an important breeding ground for the development and spread of antibiotic resistant bacteria. . An important cause of increasing antibiotic resistance is the selection of resistant bacterial strains by mutation and transfer of mobile resistance genes.Out breaks with a common source of multiple resistant bacteria ,often caused by organisms such as Pseudomonas spp,Klebsiella spp,and Acinetobacter spp are another hazard. 2,3 Klebsiella are ubiquitously present and reported worldwide. 4 In recent years Klebsiella have become important pathogens in nosocomial infections in the india. Klebsiella are also important in nosocomial infections among adult and pediatric populations. Klebsiella account for approximately 8% of all hospital-acquired infections, placing them among the top 8 pathogens in hospitals. 5 Klebsiella cause as many as 14% of cases of primary bacteremia, second only to Escherichia coli as a cause of gram-negative sepsis. 6 They may affect any body site, but respiratory infections and UTIs predominate. Mortality rates are as high as 50% and approach 100% in persons with alcoholism and bacteremia. 7 Epidemic and endemic nosocomial infections caused by Klebsiella species are leading causes of morbidity and mortality. 8 K.pneumoniae is a primary pathogen and can cause a classic form of primary pneumonia 2,9 K.pneumoniae can also cause urinary tract infection, nosocomial infections, wound and biliary tract infection, peritonitis, meningitis, bacteremia, enteritis, septicemia. 10 K oxytoca is among the top 4 pathogens that cause infection in patients in neonatal intensive care units. 11 It is the second most frequent cause of gramnegative neonatal bacteremia. Pathogens that cause infection in patients in neonatal intensive care units. It is the second most frequent cause of gramnegative neonatal bacteremia. 11

Extensive use of broad spectrum antibiotics in hospitalized patients has led to both increased

carriage of Klebsiella and subsequently the development of multidrug resistant strains that produce extended spectrum betalactamase (ESBL,AmpC) and carbapenamase (KPC,MBL).12 These strains are highly virulent, show capsular type K55 and have extra ordinary ability to spread. 13 Most outbreaks are due to single clone or single gene.The bowel is the major site of colonization with infection of urinary tract,and wounds. Bacteremia and significant increased mortality have resulted from infection with these species. 14

In India it has been reported that 65.4%isolates were ESBL producers, 28.5were AmpCproducers, 9.4%were combined ESBL and AmpC producers and 48.6%were Carbapenamases producers in which 25.6%were KPC and 23% MBL producers and 8.2%were KPC and MBL coproducers. 12,14,15 For ESBL and AmpC producers, Carbapenems remain the drug of choice,where as in carbapenem resistant strains we are left with tigecycline and polymyxins which have started developing resistance to many GNBs.Hence the detection of Carbapenem resistance is important in treatment of patients and also preventingthe spread of resistant strains. 16

The emergence and rapid spread of Multidrug resistant isolates of Klebsiella species causing nosocomial infections are of great concern worldwide. 2,17 Because of multidrug resistance of these isolates, it poses an intriguing problem to the treating clinician and increasing the mortality of the patients. 18 Hence invitro antimicrobial susceptibility pattern and identification of resistance pattern is important before treating Klebsiella infections. Therefore the present study was undertaken to assess the most prevalent species among Klebsiella infections, the prevalent antibiotic sensitivity pattern, various resistance mechanisms among the isolates and the genes involved in Carbapenem resistance.This may provide the necessary information to formulate a hospital antibiotic policy and also to prevent the spread of multidrug resistance strains in the community.

Microbial infections have been responsible for some of the most devastating events in human history. The bubonic plague, smallpox, cholera, tuberculosis and innumerable other infectious agents will forever be remembered because of the immense impact they have had on human lives. It is no wonder, then, that the advent of antibiotic drugs in the mid-20th century stands among the greatest of human achievements. However, the efficacy of drug treatments have universally waned from the moment of their introduction. Resistance mechanisms towards new antibiotics have inevitably sprung forth after their use in the market, often in the span of just a few short years (Figure 1). 19

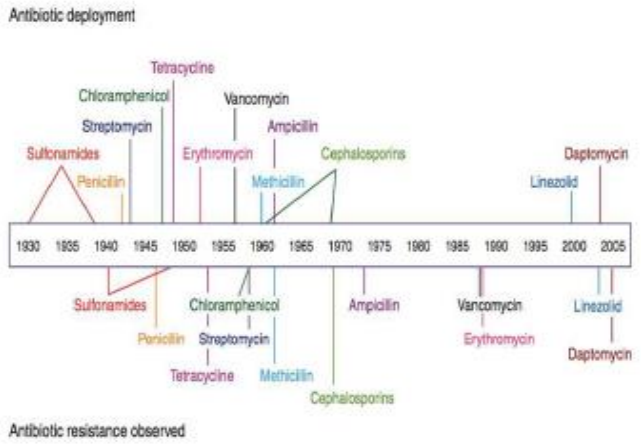

Figure 1: Timeline of antibiotic use and appearance of drug resistance.

The short efficacious lifespan of new antimicrobials has disincentivized pharmaceutical companies from investing resources in further antibiotic research and development. This has led to a critical decline in the number of new treatments available, while the prevalence of antibiotic-resistant organisms steadily rises. 20 Current trends suggest we are moving towards the emergence of a so-called "postantibiotic" era, in which we will once again lack the means to medically treat infections. The implications of this are drastic considering approximately 720,000 nosocomial infections and 75,000 resultant deaths occur every year in the United States alone. 21 Globally, 90 million people contracted malaria and 2.6 million contracted pneumonia in 2019. In fact, mortality from infectious and parasitic diseases accounts for 19.1% of global deaths, even in our current pre-"post-antibiotic" world.

As such, a new class of antimicrobial drugs would be more valuable now than at any time since prior to the discovery of penicillin. However, in view of the rate of evolutionary acquisition of resistance, it is critical that efforts be made to limit the ability for pathogens to quickly develop a means of subverting a drug's mode of action. Modern thinking asserts that molecules that interfere with microbial pathogenicity rather than survival will reduce the selective pressure that leads to antibiotic resistance, and allow natural immunity to fight off an infection. Therefore, drugs that are designed with these precepts should be capable of preventing or delaying the emergence of drug resistance.

Classification of Klebsiella

Klebsiella pneumoniae is a Gram-negative rod shaped bacteria in the Enterobactericeae family. While part of the normal flora of the human body, various strains possess high pathogenic capacity, especially while acting as a nosocomial agent and towards people with pre-existing conditions resulting in compromised immune systems (e.g. diabetes, alcoholism, HIV, etc.). Its modes of pathogenicity allow it to clinically manifest as urinary tract infections, pneumonia, septicemia and soft tissue infections. Klebsiella pneumoniae accounts for 3-7% of hospital-associated bacterial infections 22 placing it among the eight most prevalent infectious pathogens. However, the greatest concern towards K.

pneumoniae is due to the drug resistance profile of many clinical isolates. Pathogenic strains often carry plasmid-born resistances, and K. pneumoniae was among the first organisms found to possess genes for aminoglycoside resistance and extended-spectrum β-lactamase (ESBL) activity, whichconfers resistance to cephalosporins and aztreonam.23

In the early 2000's, a novel β-lactamase was found in Klebsiella, termed "Klebsiella pneumoniae carbapenemase" (KPC). KPC confers resistance to virtually all β-lactam drugs, including penicillins, cephalosporins, monobactams, and carbapenems.23 This was a critical development as carbapenems are a class of drug with regulated medical use as a "last resort" treatment for Gram-negative bacterial infections in order to preserve their efficacy and longevity. The KPC gene proved to be highly mobile and spread rapidly among other Gram-negative species, creating a class of organisms known as "carbapenem-resistant Enterobactericeae" (CRE). The KPC gene is now the most commonly found carbapenemase gene worldwide.24 In 2009, a new carbapenemase gene with a novel mode of action was isolated from K. pneumonia. 25 Named the "New Delhi metallo-β-lactamase", this gene is also rapidly spreading across the globe and among other Gram-negative bacteria such as E. coli, Enterobacter cloacae, and Salmonella enterica. A survey of New Delhi carbapenemase-bearing clinical isolates in the U.S. showed that all of them also possessed resistance to additional antibiotics including aminoglycosides and fluoroquinolones, and also possessed one or more alternate β–lactamases.26 Due to difficulty of treatment, CRE organisms are significantly more virulent, with mortality rates for septic patients at 71.9% vs. 21.9% for non-CRE infections.27 The rate of occurrence is also increasing, with the proportion of CRE infections accelerating from 0.6% to 5.6% during 2019-2020.28 K. pneumoniae currently poses a major threat to the medical community and is poised to become an even greater danger in the near future. Pathogenic strains of K. pneumoniae possesses a suite of traits that help to enable their capacity for infection and morbidity, which include: mucoviscosity, cellular adhesion, biofilms, and siderophores.

Mucoviscosity

High mucoviscosity, often referred to as hypermucoviscosity, is a common characteristic of many infectious strains of K. pneumoniae. Manifesting as an extraordinarily thick accumulation of extracellular capsular polysaccharide (CPS) surrounding the cells. Positive strains can be readily identified by a "stringiness" of the colonies (Figure 2).29 The capsule serves as a physical buffer that helps exaggerate bacterial defense mechanisms.

Figure 2: Hypermucoviscous Klebsiella pneumoniae. This positive stretch test of a hypermucoviscous strain shows the characteristic stretch of a mucoid colony.

The hypermucoviscosity trait strengthens defense against host immune responses and contributes significantly to host mortality during infection. K. pneumoniae strains of the K1 and K2 serotypes most highly associated with hypermucoviscosity express an estimated 15-17 genes implicated in upregulation of CPS synthesis. 30, 31 Of these, the magA gene (K1 strains), confers complete resistance to bactericidal human serum and a greater than 105-fold decrease to the lethal dose in mice. 32 High capsule productions in both serotypes is reported to confer resistance to opsonin-dependent phagocytosis by human neutrophils. 33 Non-capsule forming K. pneumoniae mutants showed drastically reduced virulence in mouse pulmonary infections. 34 These findings highlight the importance of capsule production as a virulence factor in K. pneumonia infections.

Multi Drug Resistant(MDR)

The isolates resistant to atleast three classes of antimicrobial agents including all penicillins, cephalosporins, fluoroquinolones and aminoglycosides.

Extensive Drug Resistant(XDR)

The isolates will be resistant to carbapenems in addition to the MDR drugs.

Pan Drug Resistant(PDR)

The isolated will be resistant to all the available drugs,including polymyxins and tigecycline.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) may be performed in several ways. The most common method used for AST in routine laboratories is conventional disk diffusion, which categorize microorganisms as S, I or R. The use of plastic strips, containing an antimicrobial concentration gradient (gradient test), is a convenient way to generate MIC data on agar plates. Broth dilution is considered the gold standard of MIC determination, but is not commonly used in routine laboratories. Recently, a standardized disk diffusion method (The CLSI method) was validated and implemented in several countries, including India. furthermore, automated AST systems (e.g. Vitek2 and Phoenix) are commonly used for AST in routine laboratories, and offer the convenience of combining species identification and MIC determination for relevant agents Introduced. Due to widespread use of broad-spectrum cephalosporins, such as ceftazidime

and cefotaxime, numerous TEM- and SHV-mutants with extended spectrum evolved.

Mechanisms of resistance

Antibacterial resistance may be intrinsic (natural) or acquired. Intrinsic bacterial resistance to antibacterial, produced by other bacteria or fungi, existed in the environment before antibacterial compounds were taken into clinical use. Bacteria, furthermore, have the remarkable ability of environmental adaptation by changing their genome through mutations or by horizontal gene transfer (HGT), or by differential gene expression. The extensive use of antibiotics in humans, but also in food animals and fish farming, has led to a selective pressure in several environmental niches promoting acquisition of resistance determinants.

The mechanism of resistance in Klebsiella involves the following three broad categories

1. Antimicrobial inactivating enzymes.

2. Reduced access to bacterial targets.

3. Point mutations that change targets or cellular functions.

Ethical consideration

Approval was obtained from the Institutional ethics committee before the commencement of the study. Informed consent was obtained from all the patients who participated in this study. All the patients satisfying the inclusion criteria were included. Patients were interviewed by structured questionnaire.

Statistical analysis

Statistical analyses were carried out using Statistical Packages for Social Sciences (SPSS). The proportional data of this cross sectional study were using Pearson's Chi Square analysis test & Fisher Exact test.

Study Population

A total of 200 clinically significant, consecutive, non-duplicate isolates of Klebsiella spp. were included in this study. The isolates were from various clinical specimens sent to the Institute of Microbiology for bacteriological culture, biochemical identification and antibiotic susceptibility testing. Isolates included in this study were obtained from blood, sputum, endotracheal aspirate, bronchial wash, pleural fluid, ascitic fluid, peritoneal dialysis fluid, cerebrospinal fluid, urine and wound swabs.

Inclusion criteria

1. Clinically significant, consecutive, non-duplicate isolates were included in the study. The significance of the isolate was based on two or more of the following criteria-clinical history, presence of organism in Gram stain, presence of intracellular forms of the organism and pure growth in cuture with a significant colony count wherever applicable.

2. Patients aged more than 18 years

Exclusion criteria

1. Isolates of repeated samples from the same patient were not included in the study.

2. Patients aged less than 18 years were not included.

Preliminary identification of isolates belonging to genus Klebisella was done based on the following characteristics.

COLONY MORPHOLOGY

On Nutrient agar

large sized greyish white with smooth surface, mucoid and opaque colonies without any pigmentation without any specific odour.

On Blood agar

mucoid circular colonies without hemolysis.

On MacConkey agar

mucoid lactose fermenting colonies.

On CLED

circular, 1-2 mm in diameter mucoid lactose fermenting colonies.

1. The isolates obtained were subjected to preliminary tests like Gram staining, Catalase test, Oxidase test and Motility by Hanging drop method.

2. The isolates which were Gram negative bacilli, catalase positive, oxidase negative and non – motile by hanging drop were subjected to biochemical reactions for further confirmation.

3. The following preliminary biochemical reactions were done with appropriate controls – Triple sugar iron agar medium for sugar fermentation and hydrogen sulphide production, Indole production using Kovac's reagent and Citrate utilization on Simmons Citrate Medium. Urease production test on Christensen's urease medium. Methyl red test for acid production and Voges proskauer test for acetoin production, lysine decarboxylation.

4. Isolates giving the following reactions were further processed in the study: by Moller's decarboxylase medium.

Triple Sugar iron agar – Acid slant /acid butt with abundant gas,no hydrogen sulphide production.

1. Indole was not formed on adding Kovac's reagent to 24hr broth culture, formed only in Klebsiella oxytoca isolates.

2. Presence of growth or change in colour from apple green to blue denotes utilization of citrate.

SPECIATION OF KLEBSIELLA ISOLATES

Phenotypic characterization

The isolates which were identified as belonging to the Genus Klebsiella were subjected to the following biochemical reactions for speciation.

1. Hugh &Leifson's OF medium:A set of semisolid medium containing 1% glucose was inoculated with a young agar slope culture. One of the tube was immediately overlaid with sterile paraffin oil to produce anaerobic condition. The species which utilizes carbohydrates both

guidelines. The control strains were included as per the CLSI guidelines.

The panel of drugs used for antimicrobial sensitivity testing

ANTIBIOTICS	RESISTANT (mm)	INTERMEDIATE (mm)	SENSITIVE (mm)
Cefotaxime (30µg)	≤22	23-25	≥26
Cefepime (30µg)	≤18	19-24	≥25
Ceftazidime(30µg)	≤17	18-20	≥21
Piperacillin-Tazobactam(100/10µg)	≤17	18-20	≥21
Amikacin (30µg)	≤14	15-16	≥17
Gentamycin (10µg)	≤12	13-14	≥15

Table 1: The panel of drugs used for antimicrobial sensitivity testing

Interpretations were made using the Clinical and Laboratory Standards Institute, USA guidelines (January 2016, M100-S24- Volume 34 No.1, Table 2B-2, page 62/63).

Minimum inhibitory concentration(MIC) by Epsilometer (E-test) method

A predefined stable antimicrobial (Imipenem) gradient is present on a thin inert non -porous plastic carrier strip 5mm wide, 60mm long known as Imipenem E-test strip. when this IMP E test strip is applied on to an inoculated agar plate , there is an immediate release of the drug and establishment of an antimicrobial concentration gradient in an agar medium. After overnight incubation, the tests are read by viewing the strips from the top of the plate,a symmetrical inhibition ellipse is produced. The intersection of the lower part of the ellipse shaped growth inhibition area with the test strip indicates the MIC value. The same MIC interpretative criteria used for dilution methods, as provided in CLSI guidelines are used with the E-strip value to assign an interpretive category of susceptible, intermediate, or resistant.

MIC INTERPRETATIVE CRITERIA FOR IMIPENEM

CATEGORY	MIC VALUE(µg/ml)
SUSCEPTIBLE	≤1
INTERMEDIATE	2
RESISTANT	≥4

Table 2: Mic Interpretative Criteria For Imipenem

RESULTS & CONCLUSION

This cross sectional study was conducted in Miracle Paramedical College, Mandsaur, with District Government Hospital, Mandsaur A total of 200 clinically signicant, consecutive nonduplicate isolates of Multidrug resistant Klebsiella species from various clinical specimens

All the isolates were identified by standard

Table 3 .procedures.

AGE IN YEARS	NO.OF PATIENTS			PERCENTAGE
	MALE	FEMALE	TOTAL	
18-20	18(12.6%)	17(11.9%)	35	17.5%
21-40	42(29.5%)	19(32.6%)	61	30.5%
41-60	70(49.2%)	19(32.6%)	89	44.5%
>60	12(8.4%)	3(5.1%)	15	7.5%
TOTAL	142(71%)	58(29%)	200	100%

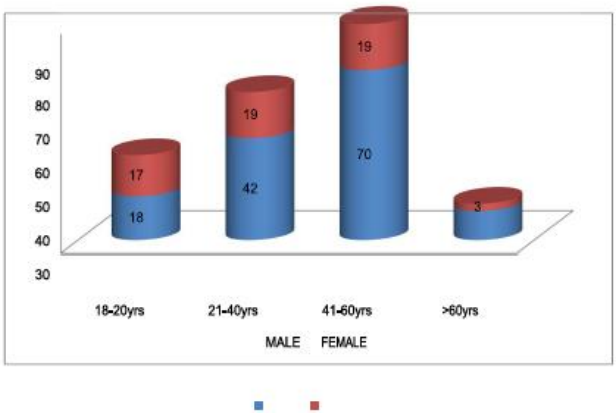

Figure 3: Gender and Age Distribution of the Patients (N=200)

CLINICAL SAMPLES	ISOLATES	PERCENTAGE
PUS	76	38%
URINE	49	24.5%
SPUTUM	24	12%
BODY FLUIDS	19	9.5%
TRACHEAL ASPIRATES	14	7%
DEVICES	12	6%
CSF	3	1.5%
BLOOD	3	1.5%

Table 4: Distribution of Klebsiella Isolates from Various Clinical Specimens (N=200)

Most of the Klebsiella isolates obtained were from pus samples (38%) followed by urine samples (24.5%) followed by sputum (12%), body fluids (9.5%), tracheal aspirates (7%), devices (6%), CSF(1.5%), blood (1.5%).

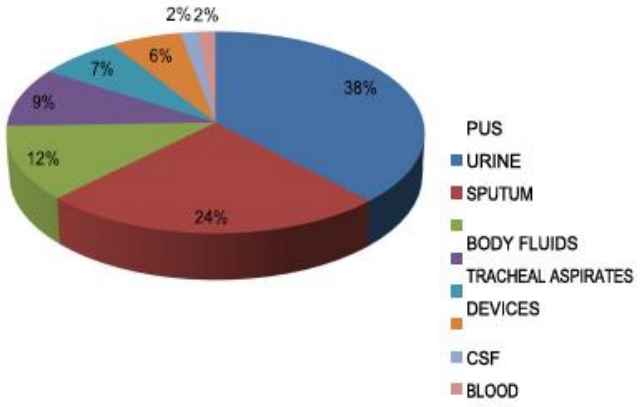

Figure 4: Distribution of Klebsiella Isolates from Various Clinical Specimens (N=200)

S.NO	SPECIES	NUMBER OF ISOLATES	PERCENTAGE
1	Klebsiella pneumonia subsp aerogenes.	96	48%
2	Klebsiella oxytoca	92	46%
3	Klebsiella pneumonia subsp pneumonia.	12	6%
	TOTAL	200	100%

Table 5: Distribution of Klebsiella Species (N=200)

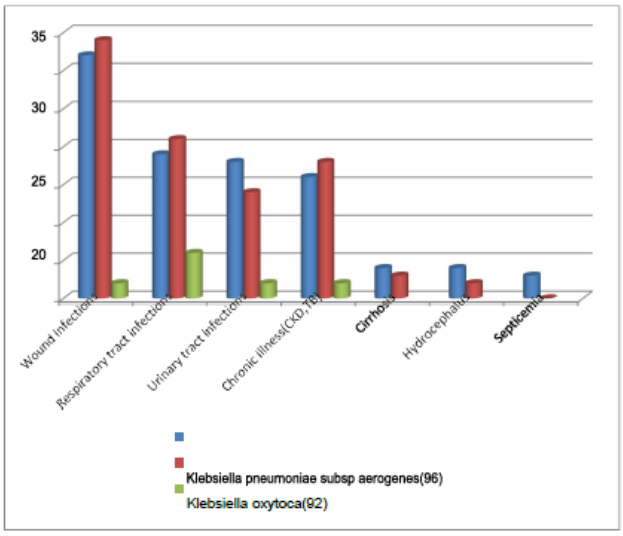

Figure 5: Distribution Of Klebsiella Species In Various Clinical Infections

Data were analysed using SPSS software (Version 10.0; SPSS Inc., Chicago). Chi-square and Fisher's exact test was performed to determine statistically significant differences among the antibiotic susceptibility rate of K. pneumonia e and Klebsiella oxytoca isolates. The standard significance level, $P < 0.05$, was used, and all tests of statistical significance were two-tailed. There was a significant difference between the

antimicrobial sensitivity pattern of Klebsiellapneumonia and Klebsiella oxytoca since p value is < 0.05 for cephalosporin, aminoglycosides,quinolones and carbapenems .

IMIPENEM SUSCEPTIBILITY	DISC DIFFUSION METHOD	PERCENTAGE
SUSCEPTIBLE	145	72.5%
RESISTANT	55	27.5%

Table 6: Detection of Imipenem Resistance In Klebsiella Species By Disc Diffusion Method(N=200)

Among the 200 isolates of Klebsiella species were screened for Imipenem resistance by Kirby-Bauer disc diffusion method of which 55 isolates(27.5%) were found to be resistant to imipenem.

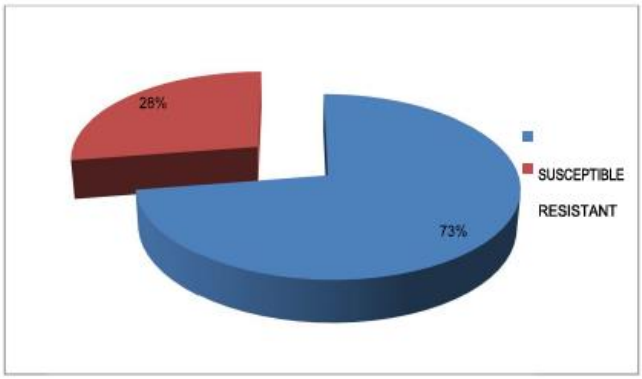

Figure 6: Detection of Imipenem Resistance in Klebsiella Species by Disc Diffusion Method

CATEGORY	MIC VALUE(µg/ml)
SUSCEPTIBLE	≤1
INTERMEDIATE	2
RESISTANT	≥4

MIC INTERPRETATIVE CRITERIA FOR IMIPENEM.

Among the 55 isolates were found to be resistant to Imipenem by Double dics diffusion method which was confirmed by MIC by E-strip method. Among the 55 isolates, 33 isolates had 32(µg/ml) as MIC, 13 isolates had 16(µg/ml) as MIC, 4 isolates had 8(µg/ml) as MIC, and remaining 5 had 4(µg/ml) as MIC.

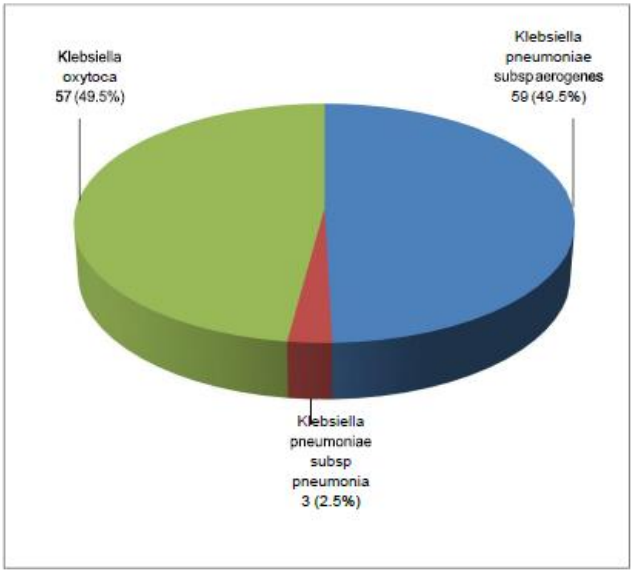

Figure 7: Detection of Extended Spectrum Betalactamases among Klebsiella Species(N=119)

Table 8: Detection of Ampc Betalactamases Among Klebsiella Species(N=13)

SPECIES	CEFOXITIN (30µg) ≤22mm	CEFOXITIN + CLOXACILLIN (30+200µg) ≥27mm	PERCENTAGE
Klebsiella pneumoniae subsp aerogenes (96)	46	8	61.5%
Klebsiella oxytoca (92)	48	5	38.5%
TOTAL	92	13	100%

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