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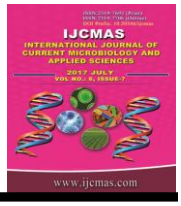

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Characterization and Antibiogram of *Klebsiella* Isolated from Clinical Samples

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A B S T R A C T

Klebsiella spp exhibits an increased antimicrobial resistance by producing Extended Spectrum Beta Lactamases (ESBL) and Carbapenamases. Studying their resistance pattern will help in appropriate use of antibiotics and infection control. Aim of the study is to characterize *Klebsiella*, to determine their antibiogram by disc diffusion, phenotypic detection of ESBL and carbapenemase. The study was conducted in the Department of Microbiology, K.S. Hegde Medical Academy, on the isolates of *Klebsiella* from samples of exudates, blood, CSF, body fluids, sputum and urine from October 2014 to April 2016. *Klebsiella* were characterized and antibiotic susceptibility testing, phenotypic tests for ESBL and Carbapenemase production were done as per CLSI guidelines. Amongst 509 *Klebsiella* isolates, 93.3% were *Klebsiella pneumoniae* and 6.6% were *Klebsiella oxytoca*. Maximum susceptibility was to Meropenem (76.6%), Imipenem (75.83%). Maximum resistance (68.5% - 69.5%) was to the third generation Cephalosporins. Multi drug resistant *Klebsiella* comprised 28%, ESBL producers were 53.83% and 12.3% were Carbapenamase producers. Monitoring the ESBL and Carbapenamase production, for an effective antibiotic policy prevents MDR *Klebsiella* and isolation by strict infection control prevents outbreaks.

Introduction

The genus *Klebsiella* contains Gram negative, capsulated, non-motile bacilli, belonging to the Enterobacteriaceae family (Mackie, 1999). There are five species under this genus, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella planticola*, *Klebsiella terrigena*, and *Klebsiella ornithinolytica*. Amongst them, the most common opportunistic and nosocomial pathogen is *Klebsiella pneumoniae* causing pneumonia, pyogenic infections, meningitis, urinary tract infections (UTI) and rarely diarrhoea and attack immunocompromised, hospitalized individuals associated with diabetes mellitus, chronic pulmonary, cardiac, renal and neoplastic diseases (Orhue *et al.*,2015)

They can be found in the gastrointestinal tract of humans and animals and have a wide distribution in nature (Koneman, 2006). They are exhibiting an increase in antimicrobial resistance making it essential for the identification of resistant bacteria. This in turn helps to tailor the empirical therapy as there will be no new antibiotics in the near future (Bora *et al.*, 2014).

This resistance of *Klebsiella* spp is mainly due to the production of extended spectrum beta lactamases (ESBLs), which are enzymes that hydrolyse and inactivate betalactam drugs like penicillin, third generation cephalosporins, aztreonam. Recent reports record ESBL producing *Klebsiella* spp being resistant to aminoglycosides, flouroquinolones, tetracycline, chloramphenicol, and sulfonamides (Asma *et al.,* 2012).

A major risk factor for ESBL producing *Klebsiella pneumoniae* is the widespread use of third generation cephalosporins. Other risk factors which contribute to colonization and infection are arterial and central venous catheterization, prolonged stay in Intensive care unit (ICU), low birth weight in preterm infants, prior antibiotic use and mechanical ventilation (Gupta *et al.,* 2003).

ESBLs are coded by transferable, conjugative plasmids which can lead to outbreaks (Shukla *et al.,* 2004). These multi drug resistant (MDR) bacteria mostly do not respond to the available antibiotics. The drug of choice for ESBL producing pathogens are carbapenems, but the increase in use is leading to selection pressure and the emergence of carbapenem resistant organisms (Lee *et al.,* 2006). It is a cause for concern as carbapenems are also the last line of treatment in multi-drug resistant *Klebsiella pneumoniae* infections (Pitout *et al.,* 2015).

There is a global spread due to the acquisition of resistance through mobile genetic elements encoding carbapenamases. Carbapenamase producing *Klebsiella pneumoniae* (CPKP) is a major nosocomial pathogen among the carbapenamase producing enterobacteriaceae (CPE) (Tseng *et al.,* 2015).

Carbapenem resistant *Klebsiella pneumoniae* is associated with high morbidity and mortality and the treatment options have been further narrowed down to polymyxins, which can be prevented by the judicious use of carbapenems (Kaur *et al.,* 2016).

Hence studying the resistance pattern of these organisms will help in guiding the appropriate use of antibiotics and in designing the antibiotic policy for infection control programmes (Ravichitra *et al.,* 2014) as the prospect of new antibiotics in near future is very less (Shweta *et al.,* 2014).

This study aimed to characterize *Klebsiella* isolated from all clinical samples, determine their antibiogram by disc diffusion method and confirm the presence of ESBL, Carbapenemase in multidrug resistant strains by phenotypic methods and the results will help in instituting infection control against these organisms.

Materials and Methods

The study was conducted in the Department of Microbiology, K.S. Hegde Medical Academy, on the isolates of *Klebsiella* isolated from samples of exudates, blood, CSF, body fluids, sputum and urine sent to the Department of Microbiology from October 2014 to April 2016.

Methods of processing

Mucoid, lactose fermenting, colonies on Mac Conkey's agar and greyish, mucoid colonies on blood agar after overnight incubation at 37ºC were subjected to standard Biochemical tests like indole production, citrate utilization, triple sugar iron, urease production, mannitol motility, sugar fermentation, aminoacid decarboxylation, methyl red and Voges-Proskauer (Table 1).

Antibiotic susceptibility testing was done on Muller Hinton agar using Kirby Bauer disc diffusion method, according to the CLSI guidelines for the following antibiotics :

Ampicillin 10 mcg, Piperacillin 100 mcg, Piperacillin/tazobactam 100/10 mcg, Cefepime 30 mcg, Cefotaxime 30 mcg, Ceftriaxone 30 mcg, Cefoxitin 30 mcg, Ceftazidime 30 mcg, Imipenem 10 mcg, Meropenem 10 mcg, Gentamycin 10 mcg, Tobramycin 10 mcg, Amikacin 30 mcg, Tetracycline 30 mcg, Ciprofloxacin 5 mcg, Gatifloxacin 5 mcg, Nalidixic acid 30 mcg, Cotrimoxazole 1.25/23.75 mcg, chloramphenicol 30 mcg, Nitrofurantoin 300 mcg.

Detection of Extended Spectrum Beta Lactamase (ESBL) production

Screening test

Initial screen test was done by disk diffusion method on Mueller Hinton Agar (MHA) using Ceftazidime 30 µg and Cefotaxime 30 µg disks. Ceftazidime zone of ≤22 mm and Cefotaxime zone of ≤27 mm were considered to be positive for ESBL screening. Quality control strain: *Klebsiella pneumoniae* ATCC 700603

Phenotypic confirmatory test

Confirmatory test for ESBL production was carried out by Disk Diffusion method using Ceftazidime 30 µg, Ceftazidime-clavulanate 30/10 µg, Cefotaxime 30 µg, Cefotaxime-clavulanate 30/10 µg. A ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanate v/s the zone diameter of the agent when tested alone was confirmed for ESBL production.

Amp C betalactamase screening

Screen test was done by disk diffusion method on Mueller Hinton Agar (MHA) and Cefoxitin 30 µg disk was placed. Cefoxitin zone of < 18 mm was considered to be positive for AmpC beta lactamase screening.

Carbapenamase screening

Screening is positive for those isolates which are either intermediate or resistant to any one of the carbapenems using Ertapenem 10 µg or Meropenem 10 µg or Imipenem 10 µg disk. Ertapenem zone of 19-21 mm or ≤18 mm and Imipenem, Meropenem zone of 20-22 mm or ≤19 mm were considered to be positive for Carbapenamase screening.

Carbapenamase confirmation

By the modified Hodge test using meropenem 10 µg. Enhanced growth = positive for carbapenemase production. No enhanced growth = negative for carbapenemase production. Statistical analysis was done using SPSS software version 21.0.

Results and Discussion

There was a total of 509, non-repeat isolates of *Klebsiella* from October 2014 to April 2016. The maximum isolates, (20.2%) were from 41-50 years age group, followed by 18.9% from 21-20 year's age group and the isolates were recovered more from males (61%).

Maximum isolates were from Exudates (48%), followed by urine samples (28%) and they were from samples from General Medicine (48.3%), followed by General Surgery (19.6%). All were lactose and dextrose fermenters, positive for lysine and negative for arginine, ornithine decarboxylation.

There were totally 475 (93.3%) *Klebsiella pneumoniae* isolates, out of which 438 (86.05%) were subspecies aerogenes, 36 (7.07%) were subspecies *pneumoniae*, 1 (0.19%) was subspecies ozaenae and 34 (6.67%) *Klebsiella oxytoca* isolates (Table 2).

In the resistance pattern, there was 100% resistance to Ampicillin, 32.61% resistance to Piperacillin/Tazobactam, 66.9% were resistant to Cefepime, 68.5% were resistant to Ceftazidime, 29.4% were resistant to Cefoxitin, 21.8% were resistant to both Imipenem and Meropenem, 26.7% were resistant to Amikacin, 37.5% were resistant to Gatifloxacin, 35.7% and 34.1% were resistant to Tetracycline and Chloramphenicol respectively, while there was 39.4% resistance to Cotrimoxazole (Table 3 and Fig. 1).

On the whole there were 143 (28.0%) isolates that were multi drug resistant (MDR).

Amongst the sensitivity pattern, there was 64.05% sensitivity to Piperacillin/Tazobactam, 30.26% were sensitive to cefepime, 28.2% were sensitive to Ceftazidime, 66.99% were sensitive to Cefoxitin, 75.8% and 76.6% were sensitive to Imipenem and Meropenem respectively, 69.74% were sensitive to Amikacin, 60.3% were sensitive to Gatifloxacin, 60.9% and 62.6% were sensitive to Tetracycline and chloramphenicol respectively while 56.9% were sensitive to cotrimoxazole.

Out of 509 isolates, 356 were positive for ESBL production by screening method (Table 4 and Fig. 2).

Out of 356 isolates positive for ESBL by screening method, there were 274 (76.9%) positive and 11 (3.08%) negative by the confirmatory method, while 71 (19.9%) isolates yielded no detectable results with any potentiation zones (Table 5).

Out of 475 *Klebsiella pneumoniae*, 259 (54.52%) were ESBL producers and out of 34 *Klebsiella oxytoca* isolates, 15 (44.11%) were ESBL producers. Out of 509 isolates, 259 (50.88%) were *Klebsiella pneumoniae* producing ESBL while 15 (2.94%) were *Klebsiella oxytoca* producing ESBL (Fig. 3).

There were 130 (25.5%) isolates which screened positive for AmpC beta lactamase production, out of which 125 (96.15%) were *Klebsiella pneumoniae* and 5 (3.84%) were *Klebsiella oxytoca* isolates.

Out of 509 isolates 119 (23.4%) were positive on screening for Carbapenamase production (Table 6).

Out of the 119 isolates positive for carbapenamase production by screening, 63 (52.9%) were positive and 56 (47.1%) were negative for carbapenamse production by the modified Hodge test, 62 were *Klebsiella pneumoniae* and 1 remaining isolate was *Klebsiella oxytoca*. Out of the 509 isolates, there were 63 (12.3%) Carbapenamase producers those are identified by Modified Hodge test (Table 7 and Figs. 4 and 5).

Klebsiella species, as a multidrug resistant, nosocomial pathogen is contributing to significant morbidity and mortality. It threatens the available treatment options and due to its high clinical prevalence, has become a cause for global concern.

In this study, we have over the duration of 18 months isolated a high number of 509 *Klebsiella* species (non-repeat) from various clinical specimens, as compared to the 100 *Klebsiella* species isolated over 22 months by Namratha *et al.,* (2015), 120 *Klebsiella* species isolated over the duration of 1 year by Chakraborthy *et al.,* (2016) and the 116 non repeated isolates, over 5 months reported by Asmaa *et al.,* (2012) and more commonly isolated in the 41-50 years age group, slightly lower than the 45-60 years age group found in the study by Namratha *et al.,* (2015) and Chakraborthy *et al.,* (2016).

Majority of the *Klebsiella* spp were isolated From exudates (48%) followed by urine (28%) which was in agreement with the studies by Namratha *et al.,* (2015), Biradar *et al.,* (2015) while in the studies by Asmaa *et al.,* (2012) and Chakraborty *et al.,* (2016). As far as Blood stream infections are concerned, 5% of the isolates were from Blood, Slightly lower than the 7% reported by Biradar *et al.,* (2015). This predominance of *Klebsiella* isolates occurring in exudates and urine corroborates the suggestion that they tend to cause more of wound infection and urinary tract infection, and the nature of these infections can be both nosocomial and community acquired. Studying the nature of the pathogen, along with its antibiogram from a particular word and its distribution among the others, helps in concentrating good infection control practices where needed.

The prevalence of *Klebsiella pneumoniae* was higher, in this study, 93%, than the prevalence of *Klebsiella oxytoca* which was 7%. Other studies too have reported higher prevalence of *Klebsiella pneumoniae* than *Klebsiella oxytoca* with 21.6% of *Klebsiella pneumoniae* and 2.4% of *Klebsiella oxytoca* by Chakraborthy *et al.,* (2016), 79% and 21% by Namratha *et al.,* (2015), 89% and 11% by Biradar *et al.,* (2015) and 65.5%, 34.5% by Asmaa *et al.,* (2012).

Table.1 Key biochemical reaction results for speciation

Indole	+	_	_	_
Citrate	+	+	+	+
MR	_	_	+	_
VP	+	+	_	_
Urease	+	+	+	_
Identification	*Klebsiella Oxytoca*	*Klebsiella pneumoniae* subsp aerogenes	*Klebsiella pneumoniae* subsp *pneumoniae*	*Klebsiella pneumoniae* subsp Ozaenae

+ = positive, - = negative

Table.2 Characterization pattern

Species	Number	Percentage (%)
Klebsiella pneumoniae or *Klebsiella pneumoniae* subsp *aerogenes*	438	86.05
Klebsiella pneumoniae subsp *pneumoniae*	36	7.07
Klebsiella pneumoniae subsp *ozaenae*	1	0.19
Klebsiella oxytoca	34	6.67

Table.3 Antibiotic susceptibility pattern

	COUNTS			PERCENTAGES		
	RESISTANT	INTERMEDIATE	SENSITIVE	RESISTANT	INTERMEDIATE	SENSITIVE
AST: Ampicillin	509	0	0	100.00%	0.00%	0.00%
Piperacillin	194	21	294	38.11%	4.13%	57.76%
Piperacillin/Tazobactam	166	17	326	32.61%	3.34%	64.05%
Cefepime	341	14	154	66.99%	2.75%	30.26%
Cefotaxime	354	7	148	69.55%	1.38%	29.08%
Ceftriaxone	351	7	151	68.96%	1.38%	29.67%
Ceftazidime	349	16	144	68.57%	3.14%	28.29%
Cefoxitin	150	18	341	29.47%	3.54%	66.99%
Imipenem	111	12	386	21.81%	2.36%	75.83%
Meropenem	111	8	390	21.81%	1.57%	76.62%
Amikacin	136	18	355	26.72%	3.54%	69.74%
Gentamicin	165	20	324	32.42%	3.93%	63.65%
Tobramycin	115	17	377	22.59%	3.34%	74.07%
Ciprofloxacin	204	13	292	40.08%	2.55%	57.37%
Gatifloxacin	191	11	307	37.52%	2.16%	60.31%
Tetracycline	182	17	310	35.76%	3.34%	60.90%
Chloramphenicol	174	16	319	34.18%	3.14%	62.67%
Nitrofurantoin	227	26	256	44.60%	5.11%	50.29%
Nalidixic acid	222	17	270	43.61%	3.34%	53.05%
Cotrimoxazole	201	18	290	39.49%	3.54%	56.97%

Table.4 ESBL screening

Test	Positive Number (%)	Negative Number (%)	Total Number
ESBL screening	356 (69.95)	153 (30.1)	509

Table.5 ESBL confirmation

Test	Positive Number (%)	Negative Number (%)	Not detected Number (%)	Total Number (%)
ESBL confirmation	274 (76.9)	11 (3.08)	71 (19.9)	356 (100)

Table.6 Carbapenamase production screening

	Carbapenamase screening	Percentage
NEG	390	76.6
POS	119	23.4
Total	509	100

Table.7 Carbapenamase production confirmation, modified Hodge test

Test	Positive Number (%)	Negative Number (%)	Total Number (%)
Modified Hodge test	63 (52.9)	56 (47.1%)	119 (100%)

Fig.1 Antibiotic susceptibility pattern

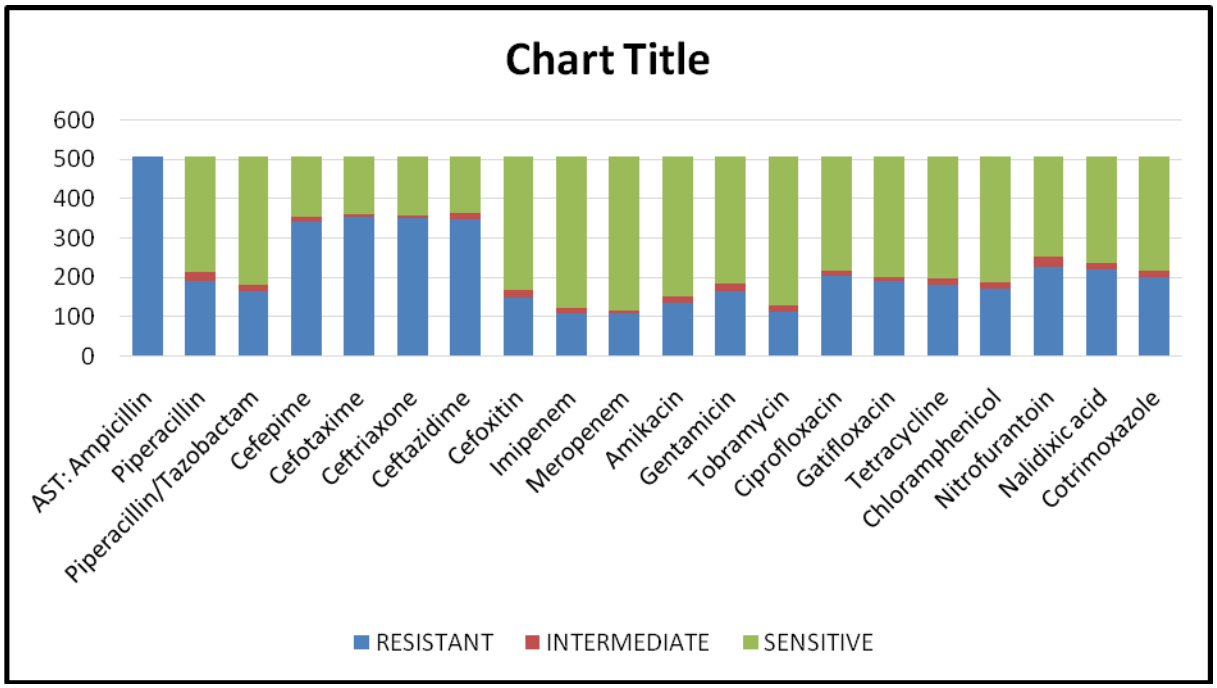


Fig.2 ESBL producers and non-producers

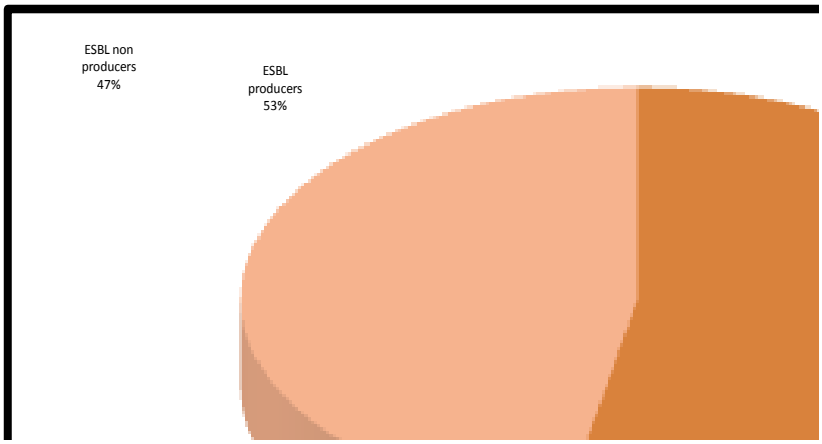


Fig.3 ESBL producers and non-producers among *K. pneumoniae* and *K. oxytoca*

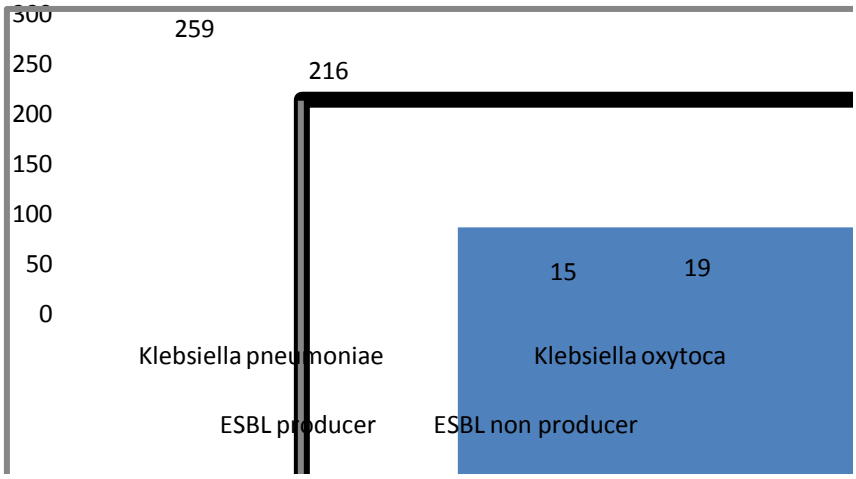


Fig.4 Modified Hodge test

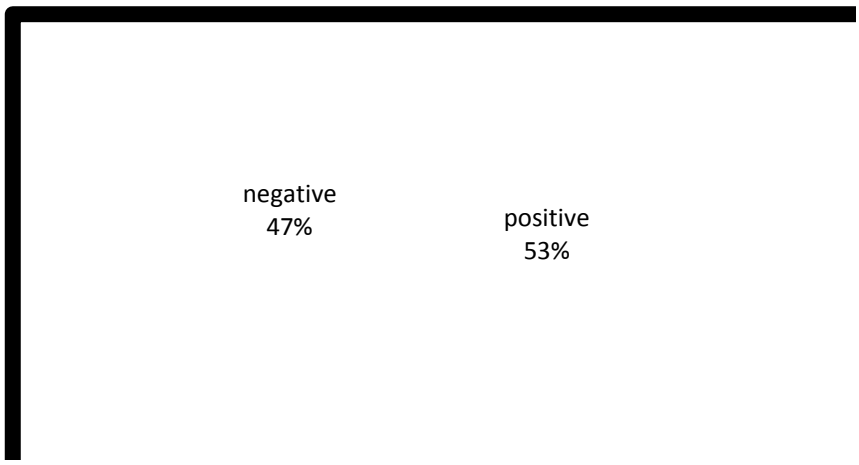
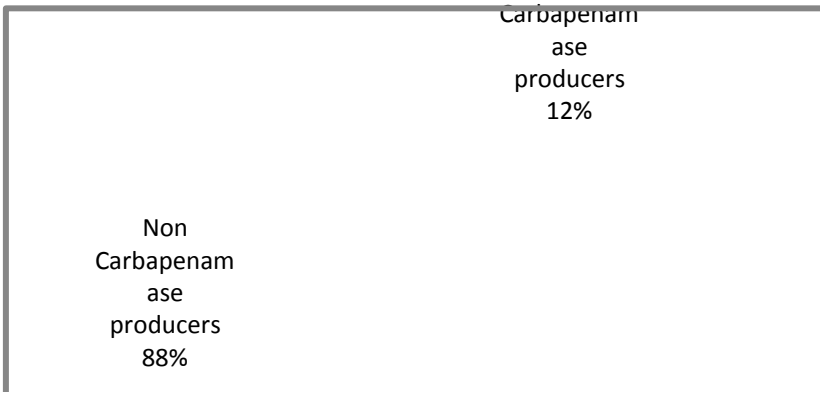


Fig.5 Carbapenamase production



Klebsiella pneumoniae was further subspeciated, with 36 (7.07%) of *Klebsiella pneumoniae* sub species *pneumoniae* 438 (86.05%) of *Klebsiella pneumoniae* subsp *aerogenes* and 1 *Klebsiella pneumoniae* subsp *ozaenae*.

The necessity to identify upto the species level helps in understanding the pathogenicity, as there are, for instance high rate of catheter tip colonization by *Klebsiella oxytoca* (Namratha *et al.,* 2015). For example, Lowe *et al.,* (2012) have reported an outbreak of *Klebsiella oxytoca* infections due to contaminated hand washing sinks, and Joainig *et al.,* (2012), have studied the cytotoxic effects of *Klebsiella oxytoca* in Antibiotic associated hemorrhagic colitis (Gupta *et al.,* 2012).

In this study, in terms of sensitivity, the maximum sensitivity was to Carbapenems, 76.6% to Meropenem followed by 75.8% to Imipenem. In terms of resistance, apart from Ampicillin, maximum resistance was observed to the 3rd generation cephalosporins (68.5% to 69.5%). In a study by Biradar *et al.,* (2015), also maximum susceptibility was to Imipinem, although it was 100% and maximum resistance (50%-70%) was observed to third generation Cephalosporins. The differences in the susceptibility pattern between this study and others suggest the nature of multiple antibiotic resistance among *Klebsiella* spp. which may be acquired through, MDR plasmids. In this study, we report 28% of the isolates as MDR, which is lower, compared to 55% MDR observed by Chakraborthy *et al.,* (2016).

These MDR organisms cause serious infections with limited antibiotics available to treat them. Continuous antibiogram evaluation is necessary to design a safe and successful empiric treatment.

The ESBL confirmation by double disk synergy test (DDST) as per CLSI guidelines, has in this study detected 53.83% ESBL producers, higher than Chakraborthy *et al.,* (2016) who reported 45%, Asmaa *et al.,* (2012), who reported 16.4% and Biradar *et al.,* (2015) who reported 24% ESBL producers.

There were 71 isolates, in which the result could not be detected, with no potentiation zone around Clavulanic acid. This may be due to Amp C betalactamase production which inhibits the inhibitor action of Clavulanic acid (Biradar *et al.,* 2015). In this study, ESBL producing *Klebsiella pneumoniae* (50.88%) was similar to 50% ESBL producing *Klebsiella pneumoniae* reported by Chakraborty *et al.,* (2016), while ESBL producing *Klebsiella oxytoca* were only 2.94% as compared to the 25% by Chakraborthy *et al.,* (2016). In the study by Asmaa *et al.,* (2012), ESBL producers among *Klebsiella pneumoniae* (11.2%) were more than for *Klebsiella oxytoca* (5.2%). The prevalence of ESBL producers vary from one region to the

other due to the differences in the infection control practices, extensive, inappropriate use of new extended spectrum antibiotics, antibiotic policy, carriage rate among hospital staff.

In this study, 119 (23.4%) isolates intermediate/resistant to Meropenem, screened positive for potential Carbapenamase production. We reported Modified Hodge Test positive for 63 isolates (12.3%) as Carbapenamase producers in this study, slightly higher when compared to 11.88% Carbapenamase producers as reported by Bora *et al.*, 2014. In a study by Mona (2016) only 1/141 (0.7%) was a Carbapenamase producer. In this study modified Hodge test has identified 63 (52.9%) as Carbapenamase producers out of the 110 Carbapenamase resistant isolates, which is lower when compared to the detection of 88.14% of Carbapenem resistant isolates in a study of Fattouch *et al.*, (2015).

The Carbapenamase resistance in isolates which were negative by Modified Hodge test could be due to impermeability by Porin loss, or over production of ESBL or Amp C beta lactamase enzyme. Many studies have reported a low sensitivity and specificity for the Modified Hodge test. This could be because this test does not differentiate between class A and class B of Caerbapenamases, but only recognizes Carbapenamase enzyme activity (Fattouch *et al.*, 2015).

MHT positive results can occur in Carbapenem resistant organisms which do not produce Carbapenamase and are not positive for all types of Carbapenamase producing organisms (CLSI, 2013). None the less, it's a simple, easy, cost effective method to detect Carbapenamase production.

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