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Original Article

Microbiology Section

Phenotypic and Genotypic Study of *Klebsiella* species with Reference to Extended Spectrum Beta-Lactamase

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ABSTRACT

Introduction: *Klebsiella* species is an important nosocomial pathogen with the emergence of Multi Drug Resistance (MDR). MDR in *Klebsiella* species is increasing worldwide with the production Extended Spectrum Beta-Lactamase (ESBL). The emergence of ESBL is a critical concern in *Klebsiella* species due to resistance to ceftazidime and other cephalosporins which compromise the efficacy of life saving antibiotics against these infections.

Aim: To know the factors responsible for antimicrobial drug resistance in *Klebsiella species* with respect to ESBL and their responsible genes.

Materials and Methods: A prospective and experimental study was carried out over a period of three years (August 2013 to July 2016). Total 200 isolates of *Klebsiella* species were screened for cefotaxime and ceftazidime. The resistant strains (cefotaxime/ceftazidime) were subjected to ESBL agar, Phenotypic Confirmatory Disc Diffusion Test (PCDDT) and Modified Three Dimensional Test (M3DT). Genetic analysis by Polymerase Chain Reaction (PCR) was done for the detection of beta-lactamase (*bla*) genes i.e., bla_{TEM}, bla_{SHV} & bla_{CTX-M} in 58 isolates of *Klebsiella* species. The data was presented using frequency and percentage. The proportion was compared using Z-test for the proportions.

Results: Out of 200 isolates, 135 (67.5%) were found resistant to cefotaxime and 125 (62.5%) were resistant to ceftazidime. Among which 110 (55%), 75 (37.5%) and 95 (47.5%) *Klebsiella* species were found positive for production of ESBL by ESBL agar, PCDDT and M3DT respectively. PCR analysis in 48 isolates were positive by PCDDT/M3DT or both were also positive for beta-lactamase genes i.e., 43 (89.58%) bla_{TEM}; 44 (91.67%) bla_{SHV} and 48 (100%) bla_{CTX-M}. Ten negative isolates either by PCDDT/M3DT or both were also negative by PCR. Co-existence of (bla_{TEM}+bla_{SHV} +bla_{CTX-M}), (bla_{TEM}+bla_{SHV}), (bla_{TEM}+bla_{CTX-M}) and (bla_{SHV}+bla_{CTX-M}) were found 81.25%, 0%, 8.33% and 10.42%, respectively.

Conclusion: The M3DT is the best phenotypic method for the confirmation of ESBL producer in *Klebsiella* species which is not included by CLSI while inclusion with PCDDT enhances the detection of ESBL producers. Co-existence of all three genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) in a single strain is a serious concern for us. So it is important to include M3DT and PCDDT in routine basis for the detection and management of ESBL producers which will help clinician to prescribe proper antibiotics.

Keywords: Antimicrobial drug resistance, Modified three dimensional test, Multi drug resistance, Phenotypic confirmatory disc diffusion test

INTRODUCTION

Klebsiella is a gram negative, encapsulated, facultative anaerobic bacterium which is opportunistic pathogen responsible for causing hospital and community acquired infections such as pneumonia, urinary tract infection, septicemia, soft tissue infections, liver abscess, meningitis etc., [1]. *Klebsiella pneumoniae* an emerging concern for the worldwide as it is one of the major causes of drug resistant nosocomial infections which are hard to eradicate using available antibiotics [2-4]. Moreover, extensive use of broad spectrum antibiotics in hospitalised patients has led to both increased carriage of *Klebsiella* and the development of MDR strains that produces ESBL. MDR strains possessing ESBLs have become an increasing problem worldwide [1] which leads to increased morbidity and mortality in hospitalised patients [4]. ESBLs enzymes are encoded by certain specific genes which are predominantly found on plasmid. They are classified into three major groups; TEM (named for a patient called Temoniera), SHV (named for sulfhydryl variable) and CTX-M (named cefotaximase as it has strong hydrolytic activity against cefotaxime; CTX as its acronym and M from Munich) [5-7]. Diagnosis of ESBL is extremely important for the proper management and treatment of infection caused by *Klebsiella* species. Few studies from Maharashtra showed production of ESBL in *Klebsiella* species as 8.5%, 24.1%, 27.6%, 28.8%, 31.75% and 90% from Mumbai, Navi Mumbai, Mumbai, Pimpri, Mumbai and Nasik, respectively [8-13]. We have very less data from our region regarding the correlation of phenotypic and genotypic expression of ESBL in *Klebsiella* species which is one of the leading pathogen causing nosocomial infection [9,12]. So this study was done to know the factors responsible for antimicrobial drug resistance in *Klebsiella* species with respect to ESBL and their responsible genes.

MATERIALS AND METHODS

A prospective and experimental study was carried out over a period of three years (August 2013 to July 2016) in microbiology laboratory of MGM Medical College and Hospital Kamothe, Navi Mumbai, Maharashtra, India. The research topic was cleared by Ethical committee for research on Human subjects on 27th October 2014 via letter no. MGM/HIS/RS/2014-15. Informed consents were taken from the patients before collection of the samples.

Inclusion criteria: Sample showing pus cells and bacteria were included in the study.

Exclusion criteria: Sample not showing pus cells and bacteria were excluded from the study.

Sample size formula used:

$$n=\frac{z^2P*q}{d^2}$$

Where,

Z=2.58 at 1% level of significance

P=the proportion=0.071

q=1-p=1-0.50=0.929

d=absolute margin of Error taken as 1%

$$n=\frac{2.58^2*0.071*0.929}{0.01^2}=4390$$

Hence, the minimum required sample size was 4390, but 4440 samples were taken [14]. Out of which, 1722 showed bacterial growth and 2718 showed no growth.

Out of 4440 samples, a total of 200 isolates of *Klebsiella* species were isolated from different clinical specimens which were characterised into different species by standard protocols [15]. The isolates were tested for their susceptibility to the Third Generation Cephalosporins (3GCs) e.g., ceftazidime (30 µg), and cefotaxime (30 µg) by using the standard disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI). If a zone diameter of <22 mm for ceftazidime and <27 mm for cefotaxime was seen then the strain was considered to be "suspicious for ESBL production" [16]. The isolates which were resistant to one of the above 3GCs were selected for the study and were further processed for the detection of ESBL production.

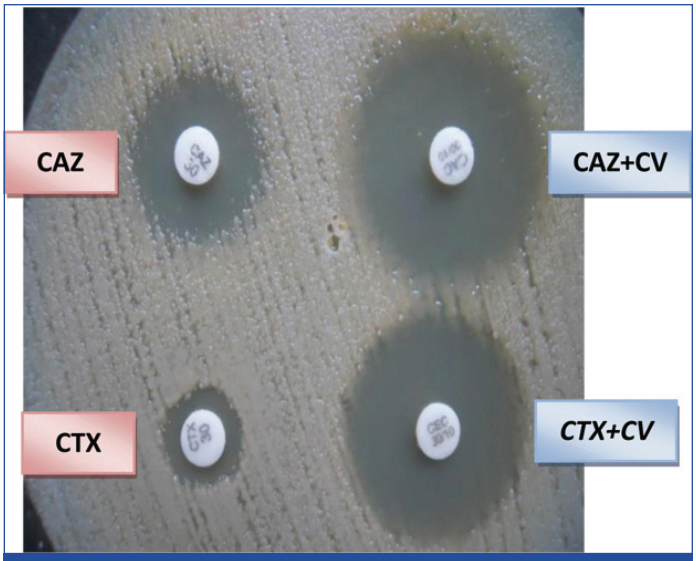
ESBL Agar

Each resistant bacterial isolates of *Klebsiella* spp was cultured on Himedia ESBL agar (Hichrome ESBL agar base and Hichrome ESBL agar supplement catalogue no. M1829 and FD278-1VL, respectively) and incubated aerobically at 37°C for 18-24 hours. Colonies of ESBL producers *Klebsiella* spp develop metallic blue colouration. Non ESBL producers grow with colourless colonies or not at all on ESBL agar as shown in the [Table/Fig-1].

[Table/Fig-1]: ESBL positive strain grown on ESBL agar.

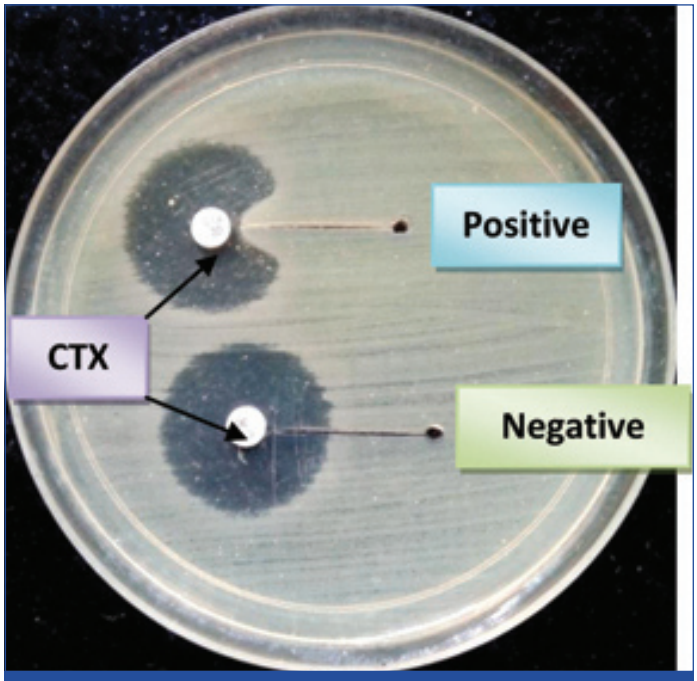
Phenotypic Confirmatory Disc Diffusion Test (PCDDT) [16]: The ceftazidime disc (30 µg) and cefotaxime (30 µg) alone and in combination with clavulanic acid (ceftazidime+clavulanic acid (30/10 µg), cefotaxime+clavulanic acid (30/10 µg) tested on Mueller Hinton Agar plate previously inoculated with the test strain. An increase of ≥5 mm in the zone of inhibition of the combination disc in comparison to the ceftazidime or cefotaxime disc alone is considered to be a marker for ESBL production as shown in the [Table/Fig-2].

Modified three-dimensional enzyme extract method (M3DT) [17]: This method was previously used for the detection of AmpC β-lactamase but here certain steps have been modified and applied



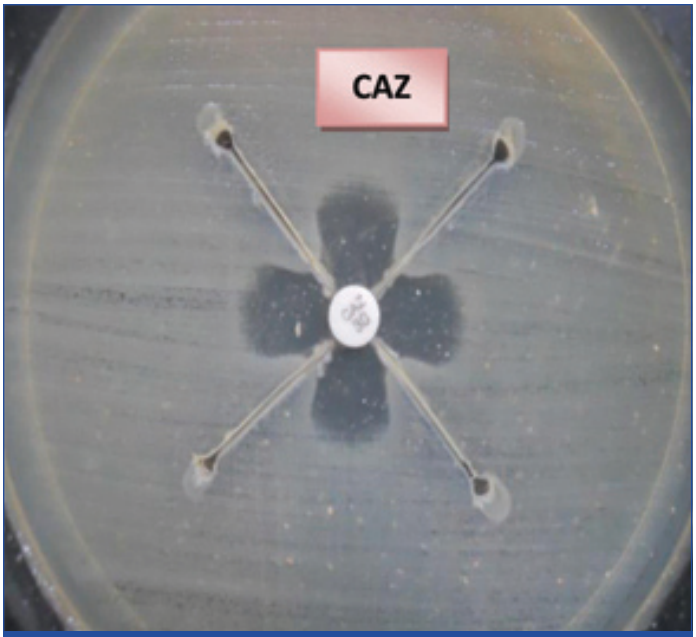
[Table/Fig-2]: PCDDT positive strains by both cefotaxime and ceftazidime clavulanic acid.
CAZ: Ceftazidime disc; CTX: Cefotaxime disc; CAZ+CV: Ceftazidime+clavulanic acid; CTX+CV: Cefotaxime+clavulanic acid

for the detection of ESBL. Overnight culture from the MacConkey agar plate was transferred into a sterile microcentrifuge tube containing peptone water and incubated at 37°C for 3-4 hours then centrifuged at 3000 rpm for 15 minutes. Afterwards, the pellet was subject to repeated freeze-thawing for several times (minimum 7) in the freezer portion of the ordinary refrigerator and crude ESBL enzyme was extracted. Ceftazidime (30 µg) and Cefotaxime (30 µg) disc were placed on Mueller Hinton agar plates containing lawn of 0.5 McFarland of *E.coli* ATCC (American Type Culture Collection) 25922 culture. Then linear slits (3 cm) was cut using sterile surgical blade, 3 mm away from antibiotic disc. A well was made using sterile standard bacteriological loop having diameter of 2 mm. A total of 30 µL of enzyme extract was loaded in the slit. The plates were kept upright for 5-10 minutes until the liquid dried and incubated at 37°C for 24 hours. The clear distortion of zone of inhibition of ceftazidime and/or cefotaxime was considered as positive test for the presence of ESBL as shown in the [Table/Fig-3,4].



[Table/Fig-3]: M3DT by Cefotaxime (CTX).

Genotypic detection [18-20]: For the Plasmid DNA Extraction, the colonies around cefotaxime disc grown on MHA were taken. Plasmid DNA was isolated by using Himedia plasmid DNA extraction kit



[Table/Fig-4]: M3DT positive for all 4 strains tested by Ceftazidime (CAZ).

Sr. No.	Genes	Primer		Amplicon size
1	bla_{TEM} [18]	F	ATAAAATTCTTGAAGACGAAA	1079bp
		R	GACAGTTACCAATGCTTAATCA	
2	bla_{SHV} [19]	F	GGGTTATTCTTATTTGTCGC	930bp
		R	TTAGCGTTGCCAGTGCTC	
3	bla_{CTX-M} [20]	F	CGCTTTGCGATGTGCAG	550bp
		R	ACCGCGATATCGTTGGT	

[Table/Fig-5]: Primers used for the PCR [18-20].

(Catalogue no. MB 508-50PR) by using manufacturer instructions. PCR analysis for ESBL genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) were carried out using following primers which were customised and procured from Eurofin Banglore [Table/Fig-5].

For PCR amplification, 5 µL of template DNA+12.5 µL of master mixture (readymade mastermix of kapa)+1 µL of forward primer+1 µL of reverse primer+5.5 µL of nuclease free water to make final volume 25 µL. Amplification was carried out according to the following thermal and cycling condition.

For TEM and SHV genes [18,19]

Initial denaturation at 94°C for	5 minutes	
Denaturation at 94°C for	1 min	35 cycles
Annealing at 58°C for	1 min	
Extension at 72°C for	1 min	
Final extension at 72°C	for 10 min	

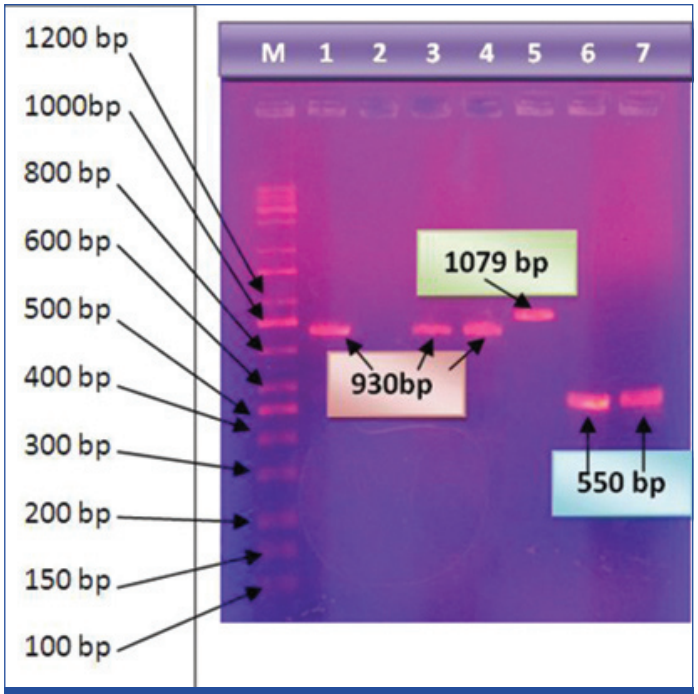
For CTX-M gene [20]

Initial denaturation at 94°C for	5 minutes	
Denaturation at 94°C for	1 min	35 cycles
Annealing at 45°C for	1 min	
Extension at 72°C for	1 min	
Final extension at 72°C	for 10 min	

The PCR products were analysed after electrophoresis in 1.5% agarose gel to detect specific amplified product by comparing with DNA ladder. After completion of electrophoresis the bands were visualised under UV transilluminator. The different amplicon size of different genes are shown in the [Table/Fig-6].

STATISTICAL ANALYSIS

The data was presented using frequency and percentage. The proportion was compared using Z-test for proportions.



[Table/Fig-6]: PCR for bla_{TEM}, bla_{SHV} and bla_{CTX-M} gene. M- Marker 100bp-3000bp. No. 1, 3 and 4 lanes amplicon of bla_{SHV}, 2 negative sample, 5 no. lane amplicon of bla_{TEM} and 6 and 7 lane amplicon of bla_{CTX-M} on agarose gel electrophoresis.

Diagnostic tests such as sensitivity and specificity were used. The level of significance was set at 5%. All p-values <0.05 were treated as significant.

RESULTS

Out of 1722 positive bacterial growth, 200 isolates of *Klebsiella* species were isolated from clinical samples with incidence rate of 11.6%. Among which 112 (56%) and 88 (44%) strains were from male and female respectively with the male female ratio 1.27:1. Out of 200, 24% (n=48) of the cases were from Outatient Department (OPD) while remaining 76% (n=152) from the Inpatient Department (IPD). Out of 200 isolates of *Klebsiella*, 135 isolates were resistant to cefotaxime and 125 isolates were resistant to ceftazidime. So they were suspicious for ESBL production, and further subjected to ESBL agar, PCDDT by using cefotaxime/clavulanic acid and ceftazidime/clavulanic acid as per CLSI guidelines and M3DT [Table/Fig-7].

Sr. No.	Method	Positive			Negative	Total
1	ESBL Agar	110 (55%)			90 (45%)	200 (100%)
2	Phenotypic Confirmatory Disc Diffusion Test (PCDDT)	Cefotaxime/ Clavulanic acid 30/10 µg	75 (37.5%)	74 (37%)	126 (63%)	200 (100%)
		Ceftazidime/ Clavulanic acid 30/10 µg		58 (29%)	142 (71%)	200 (100%)
3	Modified three dimensional enzyme extract test (M3DT)	Cefotaxime	95 (47.5%)	94 (47%)	106 (53%)	200 (100%)
		Ceftazidime		50 (25%)	150 (75%)	200 (100%)

[Table/Fig-7]: ESBL detection by different phenotypic methods.
p<0.001, Significant difference in the method and proportion of positive isolates

The incidence of ESBL producers in *Klebseilla species* in this study was 51.5% by combination of both methods i.e., PCDDT and M3DT [Table/Fig-8].

Species wise distribution of ESBL producers: Out of 200 isolates; 169 were *Klebsiella pneumoniae* subsp. *pneumoniae*, seven were *Klebsiella pneumonia ozaenae* and 24 were *Klebsiella oxytoca*. Among 169 *Klebsiella pneumoniae* subsp. *pneumoniae*, 80 (47.3%), 60 (35.5%) and 78 (46.1%) isolates were positive by ESBL agar, PCDDT and M3DT, respectively. Among seven *Klebsiella pneumoniae* subsp.

PCDDT	M3DT	*Klebsiella pneumoniae* subsp. *pneumoniae*	*Klebsiella pneumoniae* subsp. *ozaenae*	*Klebsiella oxytoca*	No. of isolates	Total Positive/ Negative
Positive	Positive	58	1	8	67 (33.5%)	Positive 103 (51.5%)
Negative	Positive	20	3	5	28 (14%)	
Positive	Negative	2	2	4	8 (4%)	
Negative	Negative	27	1	4	32 (16%)	Negative 97 (48.5%)
Cefotaxime and Ceftazidime sensitive strains i.e., Negative		62	0	3	65 (32.5%)	
Total		169	7	24	200	200

[Table/Fig-8]: Comparison of results of two diagnostic tools (PCDDT and M3DT). PCDDT: Sensitivity=72.8%; Specificity=80%

ozaenae, 5 (71.4%), 3 (42.9%) and 4 (57.1%) isolates were positive by ESBL agar, PCDDT and M3DT respectively. Among 24 *Klebsiella oxytoca*; 19 (79.2%), 12 (50%), 13 (54.17%) isolates were positive by ESBL agar, PCDDT and M3DT, respectively.

PCR: Fifty-eight isolates (48 positive and 10 negative by phenotypic methods) of *Klebsiella* from different groups were selected for the detection of genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) responsible for ESBL production. The isolates from different groups were PCDDT and M3DT positive (20), PCDDT negative and M3DT positive (20), PCDDT positive and M3DT negative (8), PCDDT and M3DT both negative (5), ESBL agar positive and PCDDT and M3DT negative (5). Co-existence of (bla_{TEM}+bla_{SHV}+bla_{CTX-M}), (bla_{TEM} +bla_{SHV}), (bla_{TEM} +bla_{CTX-M}) and (bla_{SHV} +bla_{CTX-M}) were found 81.25%, 0%, 8.33% and 10.42%, respectively [Table/Fig-9-11].

PCDDT	M3DT	No. of isolates taken	bla_{TEM}	bla_{SHV}	bla_{CTX-M}
Positive	Positive	20	20 (100%)	17 (85%)	20 (100%)
Negative	Positive	20	15 (75%)	19 (95%)	20 (100%)
Positive	Negative	8	8 (100%)	8 (100%)	8 (100%)
Total		48	43 (89.58%)	44 (91.67%)	48 (100%)

[Table/Fig-9]: Results of PCR for ESBL in PCDDT and M3DT test.

PCDDT	M3DT	No. of isolates taken	bla_{TEM}	bla_{SHV}	bla_{CTX-M}
Negative	Negative	5	0	0	0
ESBL Agar positive and (PCDDT & M3DT Negative)		5	0	0	0
Total		10	0	0	0

[Table/Fig-10]: Results of PCR for ESBL by both tests.

PCDDT	M3DT	No. of isolates taken	bla_{TEM}+bla_{SHV} + bla_{CTX-M}	bla_{TEM} + bla_{SHV}	bla_{TEM} + bla_{CTX-M}	bla_{SHV}+ bla_{CTX-M}
Positive	Positive	20	17 (85%)	0	3 (15%)	0
Negative	Positive	20	14 (70%)	0	1 (5%)	5 (25%)
Positive	Negative	8	8 (100%)	0	0	0
Total		48	39 (81.25%)	0	4 (8.33%)	5 (10.42%)
p-value		-	<0.001**	-	-	-

[Table/Fig-11]: Co-existence of genes in ESBL producing isolates. **→ highly significant

DISCUSSION

Increasing resistance to 3GCs has become a serious concern among pathogens causing nosocomial infections. The prevalence of ESBL is a critical concern to the available beta-lactam antibiotics causing treatment failure which leads to increase hospital stay and treatment costs. Among available wide range of antibiotics, beta-lactam antibiotics are widely used for almost more than 50% of the all systemic bacterial infections [21].

Knowledge of ESBL strains is necessary for proper treatment and management of patients. ESBL production in *Klebsiella* species was studied by various researchers in which incidence of it ranges from 8.5-90% [8,13]. In present study, 37.5% and 47.5% isolates of *Klebsiella* species were ESBL producer by PCDDT and M3DT respectively. Some results were similar to present study, where others reported lower or higher values which is shown in the [Table/Fig-12] [22-37]. These differences could be due to various reasons like methodology followed by different researchers (Disc approximation method, combined disc diffusion method, phenotypic disc diffusion test, E-test etc.,), constant use of particular antibiotic can lead to development of drug resistance by ESBL which will depend on the quantum of drug prescribed- can be directly proportional to prescription polices.

Author	Place	Publication year	No. of isolates	PCDDT (%)	bla_{TEM} (%)	bla_{SHV} (%)	bla_{CTX-M} (%)
Bora A et al., [23]	Assam	2014	219	67.24	80	50.57	62
Chandra V et al., [24]	Ahmedabad	2014	76	65.75	80	96	-
Khosravi AD et al., [25]	Iran	2013	55	47.27	34.61	46.15	26.92
Sharma M et al., [26]	Jaipur	2013	179	67.04	75	60	85
Mohammad MF et al., [27]	Iran	2010	89	69.7	54	67.4	-
Sobhan G et al., [28]	Iran	2011	113	59.2	16.4	94	23.9
Al-Agamy Mohammad HM et al., [29]	Saudi Arabia	2009	400	55	84.1	97.3	34.1
Babypadmini S et al., [30]	Tamil Nadu	2004	58	40	-	-	-
Krishnamurthy V et al., [22]	Karnataka	2013	87	32.1	14.81	22.22	48.5
Tawfik AF et al., [31]	Saudi Arabia	2015	430	25.6	70.9	89.1	36.4
Chandra A and Shrestha CD [32]	Nepal	2013	145	16.55	-	-	-
Kiratisin P et al., [33]	Thailand	2008	1001	12.7	71.7	87.4	99.2
Kamatchi C et al., [34]	Chennai	2009	188	-	73	42	75
Ahmed OB et al., [35]	Saudi Arabia	2013	19	-	58	63	68.4
Moosavian M and Deiham B [36]	Iran	2012	97	45.4	48.5	23	0
Shahcheraghi F et al., [37]	Iran	2007	145	34.48	32.1	69.6	-
Present study	Navi Mumbai	2021	200	37.5	89.58	91.67	100

[Table/Fig-12]: Comparative studies of ESBL from different regions of the world [22-37].

The genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity [22]. The ESBL producers usually consist of multiple resistant plasmid mediated genes e.g., bla_{TEM}, bla_{SHV} and bla_{CTX-M}. In present study, it was found bla_{TEM} 43 (89.58%), bla_{SHV} 44 (91.67) and bla_{CTX-M} 48 (100%) which was compared with other researchers given in the [Table/Fig-12] [22-37]. In present study, the best phenotypic method for the confirmation of ESBL were shown by M3DT in comparison to PCDDT; as all the negative strains by PCDDT and positive by M3DT were found positive for the ESBL genes by PCR.

The co-existence of ESBL genes in a single isolates were 39 (81.25%) for bla_{TEM}+bla_{SHV}+bla_{CTX-M}, 4 (8.33%) for bla_{TEM}+bla_{CTX-M} and 5 (10.42%) for bla_{SHV}+bla_{CTX-M}. Similar results have been reported by Bora A et al., Chandra V et al., and Khosravi AD et al., [23-25]. The presence of multiple genes in a single isolates

like bla_{TEM}+bla_{SHV}+bla_{CTX-M} may be related to complex antimicrobial resistance [22]. So it is important to continuously monitor resistance pattern and enhancing the infection control for these strains in healthcare units.

Limitation(s)

Advance molecular methods for the characterisation of *Klebsiella* species were not conducted. PCR in all cefotaxime/ceftazidime resistant isolates of *Klebsiella* for detection of ESBL genes were not conducted due to lack of funding as it was self sponsored.

CONCLUSION(S)

To conclude this, it is suggested that M3DT is best phenotypic methods for the detection of ESBL than PCDDT using both antibiotics i.e., cefotaxime and ceftazidime. The result seems to have 100% sensitive and specific by PCR. Some isolates positive by ESBL agar and negative by M3DT and PCDDT were also found negative by PCR so present study suggests not to use ESBL agar for the detection of ESBL. Bla_{CTX-M} was a dominant gene in *Klebsiella* species for ESBL producer. Increasing existence of bla_{CTX-M} and co-existence of all the three responsible ESBL genes (bla_{TEM}+bla_{SHV}+bla_{CTX-M}) are a serious concern for us. So, it is important to continuously monitor resistance pattern and enhancing the infection control for these strains in healthcare units. It is also important to report ESBL producers on routine basis which will help the clinicians to prescribe proper antibiotics to manage infections caused by them.

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