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Original Article

Microbiology Section

Prevalence of Extended Spectrum Beta-lactamase Encoding Genes in Enterobacteriaceae Isolated from Various Clinical Samples in a Tertiary Care Cancer Centre, Kerala, India

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ABSTRACT

Introduction: Cancer patients are disproportionately at high risk of developing infections, with a risk of infection about 10 times than that of non cancer patients. Extended Spectrum Beta-Lactamases (ESBLs) enzyme producing gram-negative bacteria have been marked as one of the "serious" threat by the centre for disease control. This enzyme has the ability to hydrolyse the beta-lactam antibiotics and posses a major threat for the immunocompromised and cancer patients. Hence, understanding its prevalence at the grammatical level is important to decide upon the type of drugs to be prescribed.

Aim: To study the prevalence of ESBL genes among the Gram-Negative Bacteria (GNB) isolated from cancer patients.

Materials and Methods: The present study was a hospital-based cross-sectional study carried out in Microbiology Division, Malabar Cancer Centre, Thalassery, Kerala, India, from January to March 2021. Microbiological identification of the causative agents was done by staining, culturing and biochemical methods. Screening of the isolates for ESBL was done using Double Disc Synergy Test (DDST). Antibiotic susceptibility testing was carried out using modified Kirby-Bauer disc diffusion method. The ESBL producers were genotypically confirmed through Polymerase Chain Reaction (PCR) and typed accordingly. The data were given as average with standard deviation and analysed using Microsoft Excel.

Results: Out of 1,310 specimens, 366 (27.9%) were culture positive. Among the GNB, *Escherichia coli* (50%) followed by *Klebsiella pneumoniae* (46.07%), *Proteus* (1.96%) and *Enterobacter cloacae* (0.98%) were the predominant isolates. Most of these ESBL producers (*Escherichia coli*, *Klebsiella pneumoniae*) were Multidrug Resistant (MDR). However, significant isolates of *Escherichia coli* (98.03%), *Klebsiella pneumoniae* (36.1%), *Proteus,* (50%) and *Enterobacter cloacae* (100%) were sensitive to immunocompromised. Among 102 ESBL producers, prevalence of bla_{TEM} (67.64%) was highest followed by bla_{CTX-M} (10.78%), bla_{SHV} (14.70%) and bla_{OXA} (18.62%). All of the ESBL producers tested showed the presence of one of four beta-lactamase encoding genes by PCR. One of the isolate *Proteus mirabilis* found to be ESBL producer as confirmed by phenotypic methods but lacked ESBL genes.

Conclusion: Higher prevalence of ESBL producing strains warrants stringent measures to tackle the spread of MDR strains. Carbapenems can be considered as the drug of choice against ESBL producers. Highly prevalent blaTEM gene could be considered as potential therapeutical and diagnostic target against the ESBL producing GNBs.

Keywords: Antibiotics, Cancer patients, Gram negative bacilli, Immunocompromised

INTRODUCTION

The ever-increasing bacterial resistance to antibiotics is one of the most challenging tasks that the medical world facing today. Owing to its broad spectrum and safety nature β-lactam antibiotics were preferred profusely [1]. However, resistance to the same is being emerged in recent times especially in GNB pathogens like *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. [2]. The GNB producing ESBL has emerged as a significant challenge to undertake with the present antibiotics. The bacteria which are responsible for causing infections have more aggressive virulence factors that enhance their host cell attachment, colonisation as well as virulence. A single mutation in bacteria leads to a new resistance mechanism against various drugs and wipes all the decade year scientific efforts and drags everyone to zero point again [1].

The ESBLs producing bacteria have been marked as one of the "serious" threat by centre for disease control [2]. The ESBL are the enzyme produced by GNB; that has the ability to hydrolyse various β-lactam antibiotics. Resistance is also conferred by changes in the substrate spectra [1]. This has resulted in increased morbidity, mortality, higher cost of treatment and longer hospital stay [3]. Among Enterobacteriaceae different species of bacteria are also capable of producing ESBLs *Pseudomonas aeruginosa, Klebsiella oxytoca, Proteus mirabilis, Salmonella enteric, Neisseria gonorrheae, Haemophilus influenza, Kluyvera species, Enterobacter aerogenes*, and *Enterobacter cloacae*. The ESBLs have also been reported in *Acinetobcterspp*, *Alcaligenes fecalis*, *Burkholderia* spp. [2,3]. Plasmids mediated ESBL resistance may also accompany resistance to additional β-lactamase genes while conferring resistance to other drugs of choice [3-5]. Therefore, phenotypic detection and characterisation of ESBL genes among Enterobacteriaceae species is vital to understand the spread of resistance mechanism. The present study aims to study the prevalence of ESBL genes from Enterobacteriaceae isolates among the cancer patients in our geographic region of interest.

MATERIALS AND METHODS

It was a hospital-based cross-sectional study carried out in Microbiology Division, Malabar Cancer Centre, Thalassery, Kerala,

India, on 1,310 specimens among cancer patients from medical, surgical and allied super-specialty units. The study was approved by the Institutional Review Board (IRB No: 1616/IRB-SRC/13/MCC/20-02-2021/5), of Malabar Cancer Centre. The factors like gender, age, date of admission etc., were also recorded. This study was carried out for three months from January to March 2021.

Study Procedure

Specimen collection: All specimens (Urine, sputum, Bronchoalveolar Lavage [BAL], Tracheal aspirates, stool, wound swab, suction tips, pus, all body fluids, tissues and throat swabs) received in the microbiology lab were included in the study. All specimens received in the lab were inoculated on Blood Agar, MacConkey agar, and Chocolate agar using a calibrated loop. The inoculated culture plates were incubated at 37°C for 18-24 hours. After reading and interpreting the culture results, the clinically significant isolates were identified as per the manual of clinical microbiology.

Antibiotic susceptibility testing: Bacterial isolates from the specimes were tested for antibiotic susceptibility using Kirby-Bauer disc diffusion method according to Clinical Laboratory Standards (CLSI) guidelines [6]. A panel of antibiotic discs (HiMedia) ampicillin, piperacillin tazobactam, levofloxacin, ciprofloxacin, norfloxacin, nitrofurantoin, cefuroxime, cefotaxime, ceftazidime, imipenem, meropenam, and amikacin were used for the susceptibility test. Antibiotic discs were placed onto inocluated plates, inverted and incubated at 37°C. Zones of complete inhibition was measured in millimeter post 16-18 hours of inoculation. The results were interpreted as sensitive or moderately sensitive or resistant to the antimicrobial agents, comparing with CLSI guidelines [6]. *Escherichia coli* ATCC no: 25922 was used as the quality control strain for antibiotic susceptibility test.

Double Disc Synergy Test (DDST): The DDST was done to screen the islolates for ESBL producers on Muller-Hinton Agar (MHA) using third-generation cephalosporin (Ceftazidime and cefotaxime). A minimum of three to five well-isolated colonies of the same morphological type were selected from an agar plate culture and inoculated into a tube containing 4-5 mL of a suitable broth medium such as peptone water. The culture broth was incubated at 37°C overnight. The turbidity of the actively growing broth culture was adjusted with sterile saline to 0.5 McFarland's standard and swabbed onto the agar plates ensuring even distribution of inoculums. Amoxicillin-clavulanate discs were placed in the center of the plate. The cefotaxime and ceftazidime discs were placed 15 mm and 20 mm apart, respectively, center to center to that of the amoxicillin-clavulanate disc. The plates were inverted and placed in an incubator set to 37°C. After 16-18 hours of incubation, each plate was examined. A clear-cut enhancement or synergy of inhibition between cephalosporin disc and Beta-lactamase inhibitor disc were interpreted as positive for ESBL production. All strains positive for ESBL production were stored at -20°C for further analysis [1].

Genotypic characterisation of ESBL producing GNBs for ESBL genes: Genomic DNA from each isolates was extracted using the boiling method. In brief, a single bacterial colony from a freshly streaked plate was picked and suspended into 100 µL PCR grade watertaken in 1.5 mL microcentrifuge tube. It was boiled for 10 minutes at 100ºC in dry bath. Tubes were then centrifuged at 12000 rpm for 6-7 minutes. The supernatant was used as template for PCR and subjected to multiplex PCR to target [Table/Fig-1]. The PCR programme is given in the [Table/Fig-2]. After completion of the Polymerase chain reaction, the amplified PCR product was observed by resolving the PCR products through 2% agarose gel electrophoresis [7,8]. Specific primers used in the study were purchased commercially [8,9].

ESBL genes	Nucleotide sequence (5'-3')	Product size
TEM	CATTTCCGTGTCGCCCTTC CGTTCATCCATAGTTGCCC	800 bp
SHV	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713 bp
OXA	GGCACCAGATTCAACTTTCAG GACCCCAAGTTTCCTGTAAGG	564 bp
CTX-M	TTGTTAGGAAGTGTGCCG GGCTGGGTGAAGTAAGTG	569 bp

[Table/Fig-1]: List of ESBL gene and its specific primers.

Step	Process	Temperature	Time (TEM and CTX-M	Time (SHV and OXA)
I	Initial denaturation	94°C	5 minutes	10 minutes
II	Denaturation	94°C	30 seconds	40 seconds
III	Annealing	60°C	30 seconds	40 seconds
IV	Extension	72°C	50 seconds	10 seconds
			35 cycles	**30 cycles**
V	Final extension	72°C	5 minutes	7 minutes
End or 4°C (maintained)				

[Table/Fig-2]: PCR programme for selected ESBL genes.

STATISTICAL ANALYSIS

Data were read and recorded by the authorised personnel for the confidentiality. Data were analysed using Microsoft Excel. The prevalence of each genes were confirmed by repeating the experiments in duplicates. Prevalence of specific genes out of total number of sample was expressed in terms of percentage.

RESULTS

During the study period, out of 1,310 samples collected in the Microbiology Division 366 (27.9%) were observed to be bacteriologically positive. Out of 366 positive cultures, 273 (74.5%) were GNB [Table/Fig-3]. However, prevalence were higher among female, 52.9% than male, 47.45% [Table/Fig-4].

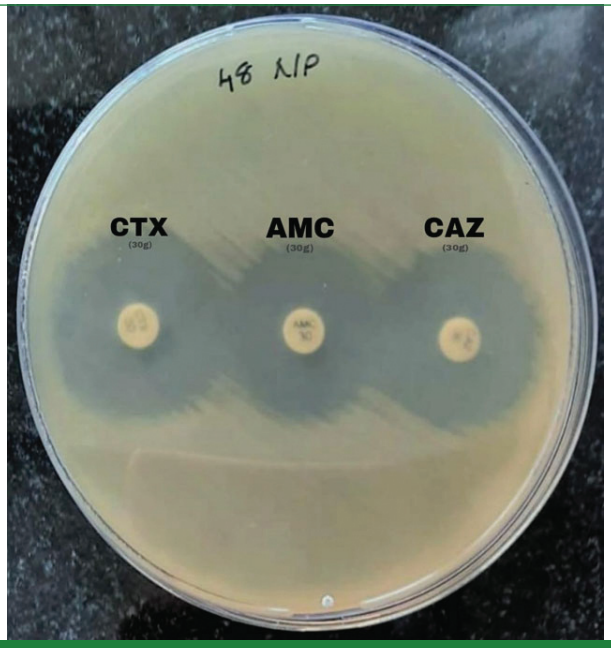
Pathogens	Total number of isolates (n=366)	Percentage
Gram positive cocci	89	24.3%
Fungal	4	1.92%
Gram negative bacilli	273	74.5%

[Table/Fig-3]: Microbial aetiology of specimens: organism based on groups.

	Escherichia coli		*Klebsiella pneumoniae*		*Proteus* spp.		*Enterobacter cloacae*		*Enterobacter aeruginosa*	
Age (In Years)	F	M	F	M	F	M	F	M	F	M
0-10	2	4	0	3	0	0	0	0	0	0
11-20	2	1	6	1	0	0	0	0	0	0
21-30	0	0	2	0	0	1	0	0	0	0
31-40	3	0	7	0	0	0	0	0	0	0
41-50	2	4	5	4	0	1	0	0	0	0
51-60	8	3	5	3	0	0	0	0	1	0
>60	8	14	3	8	0	0	0	1	0	0
Total	25 (49.01)	26 (50.9%)	28 (59.57%)	19 (40.2%)	0	2 (100%)	0	1 (100%)	1 (100%)	0

[Table/Fig-4]: Age and gender wise distribution of patients with ESBL producing Enterobactericeae (N=102).
F: Female; M: Male

Among the GNB, *Escherichia coli* was more predominant (50%) in causing infections followed by *Klebsiella pneumoniae* (46%), *Proteus* (1.96%), *Enterobacter cloacae* (0.98%) and *Enterobacter aeruginosa* (0.98%). The incidence of the ESBL producing Enterobacteriaceae among cancer patients showed that ESBL producers can cause infection at any stage and gender [Table/Fig-4]. The ESBL production were confirmed by DDST method [Table/Fig-5]. Among the ESBL producers 50% were *Escherichia coli*, while 46% were *K. pneumoniae*, 1.96% *Proteus*, 0.98% *Enterobacter cloacae*, 0.98% *Enterobacter aeruginosa*, respectively [Table/Fig-6]. The finding shows *E. coli* and *K. pneumoniae* as core of the predominant bacterial pathogen causing ESBL in cancer patients.



[Table/Fig-5]: DDST Method: Representative image showing ESBL producing GNB using DDST method.

Pathogen	Total number of isolates (n=102)	Percentage
Escherichia coli	51	50%
Klebsiella pneumoniae	47	46%
Proteus spp.	2	1.96%
Enterobacterclocae	1	0.98%
Enterobacter aeruginosa	1	0.98%

[Table/Fig-6]: Aetiology of ESBL producers.

Mostly all of the ESBL producers were MDR [Table/Fig-7]. However, significant isolates *Escherichia coli* (98.03%), *Klebsiella pneumoniae* (36.1%), *Proteus* (50%) and *Enterobacter cloacae* (100%), *Enterobacter aeruginosa* (100%) were sensitive to imipenem.

Genotypic characterisation of ESBL producers: The ESBL producing isolates were phenotypically confirmed by molecular methods (PCR) to understand the frequency of occurrence of ESBL genes. The amplified Deoxyribonucleic Acid (DNA) bands of the bla CTX-M, blaTEM, blaSHV and blaOXA genes were analysed upon Ultraviolet (UV) trans-illumination. Among 102 ESBL producers, prevalence of blaTEM (67.64%) were highest, followed by bla CTX-M (10.78%), bla SHV (15.68%) and bla OXA (18.62%) [Table/Fig-8,9]. All of the ESBL producers tested showed the presence of one of four β-lactamase encoding genes by PCR.

Pathogen	TEM	CTX-M	SHV	OXA
Escherichia coli	39	5	2	9
Klebsiellapneumoniae	27	5	13	9
Proteus	2	-	1	-
Enterobacter cloacae	1	-	-	1
Enterobacter aerogenes	-	1	-	-
Total	69 (67.64%)	11 (10.78%)	16 (15.68%)	19 (18.62%)

[Table/Fig-8]: Distribution of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA} genes.

[Table/Fig-9]: Detection of TEM and CTX-M type genes by using multiplex PCR method (a) Lane 1 shows amplification of CTX-M and Lane 2 shows amplification of TEM).

Though, the number of ESBL is 102, in certain isolates, coexpression of genes were found. This is reflected in the total number of ESBL genes.

DISCUSSION

Cancer patients undergoing chemotherapy gets repeated infections due to immune suppression. The ESBL producing organisms have rapidly spread all over the world which is attributed to overuse of expanded spectrum cephalosporin [4]. Accurate and precise detection of ESBLs is necessary for optimum antibiotic therapy,

Antibiotics	*Escherichia coli* (n=51)		*Klebsiella pneumonia* (n=47)		*Proteus* (n=2)		*Enterobacter cloacae* (n=1)		*Enterobacter aeruginosa* (n=1)	
	S	R	S	R	S	R	S	R	S	R
Amikacin	42 (82.3%)	9 (17.64%)	23 (48.9%)	24 (51%)	1 (50%)	1 (50%)	1 (100%)	-	1 (100%)	-
Amoxyclav	33 (64.7%)	18 (35.29%)	13 (27.7%)	34 (72.3%)	-	2 (100%)	-	1 (100%)	1 (100%)	-
Cefepime	17 (33.3%)	34 (66.6%)	11 (23.4%)	36 (76.5%)	-	-	-	1 (100%)	-	1 (100%)
Cefuroxime	5 (9.8%)	46 (90.1%)	9 (19.14%)	38 (80.85%)	-	2 (100%)	-	1 (100%)	-	1 (100%)
Ceftriazone	6 (11.7%)	45 (88.2%)	9 (19.14%)	38 (80.85%)	-	2 (100%)	-	1 (100%)	1 (100%)	-
Gentamycin	40 (78.4%)	11 (21.5%)	15 (31.91%)	32 (62.74%)	1 (50%)	1 (50%)	1 (100%)	-	1 (100%)	-
Imipenem	50 (98.03%)	1 (1.96%)	17 (36.17%)	30 (63.8%)	1 (50%)	1 (50%)	1 (100%)	-	1 (100%)	-
Ertapenem	48 (94.11%)	3 (5.88%)	18 (38.2%)	29 (61.7%)	2 (100%)	-	1 (100%)	-	1 (100%)	-
Meropenem	49 (96.07%)	2 (3.92%)	22 (46.8%)	25 (53.1%)	-	-	1 (100%)	-	1 (100%)	-
Pip-taz	42 (82.35%)	9 (17.6%)	16 (34%)	31 (65.9%)	2 (100%)	-	1 (100%)	-	1 (100%)	-
Cefoperazone	41 (80.39%)	10 (19.60%)	20 (42.5%)	27 (57.4%)	-	-	1 (100%)	-	1 (100%)	-
Colistin	49 (96.07%)	2 (3.92%)	44 (93.6%)	3 (6.38%)	1 (50%)	1 (50%)	1 (100%)	-	1 (100%)	-

[Table/Fig-7]: Antibiotic susceptibility testing of ESBL producing Enterobacteriaceae.

infection control measures and surveillance purposes. However, it is not happening in reality in many microbiology units of developing countries including India [6]. In recent years, bacterial resistance to these drugs has increased dramatically with ESBL contributing to this increase. Raising grave fear, a high incidence of drug resistance is noted among cancer patients through this study among which 37.36% of gram negative bacilli were ESBL producers.

The ESBLs producing GNB especially *Escherichia coli* and *Klebsiella pneumoniae* causing infection in cancer patients have emerged as serious pathogens both in hospital and community infection worldwide [7]. In the present study also, the prevalence of ESBL producing gram negative bacilli were 37.36%. Its emergence becomes more prevalent in the case of *Escherichia coli*. The underlying risk factors which are associated with ESBL production includes age [8,9]. Studies have shown that the elderly (>60) were more affected. In the present study also elderly (>60) patients were more affected 34.9%. Present observations on prevalence of ESBL producing Enterobacteriaceae among paediatric and elder cancer population are in coherence with previous ones from various geographical locations [9-11]. However, present findings were lower (8.7%) compared to a study conducted in Burkina Faso (50.8%) [11]. Also, in present study, females were more affected (53%) compared with males.

The antibiotic susceptibility pattern of all ESBL producers showed that mostly all ESBL producers (*Escherichia coli, Klebsiella pneumonia, Proteus* and *Enterobacter cloacae*) were MDR. In present study, the highest resistance level of resistance was seen to cefuroxime (90.1%). However, significant isolates were sensitive to carbapenems. Hence, it can be considered as the drug of choice as reported previously in studies from European countries that claimed these Carbapenems, such as imipenem, ertapenem, and meropenem for treatment of infection cases caused by ESBL in cancer patients [12].

The prevalence of ESBLs and its type varies within different geographic region, the same was observed in the present study too, *Escherichia coli* (50%) were the predominant pathogen followed by *Klebsiella pneumoniae* (46%). In China TEM gene was highly prevalent among ESBLs producing *E. coli* followed by SHV and CTX-M-type [13]. It was the case in India also till 2000, however CTX-M replaced TEM later [3,14]. But in Canada SHV predominated other two genes [15]. Group 2 CTX-M enzymes were prevalent in parts of South America and Israel while group 9 enzymes were found to be prevalent in Spain [16,17].

Goyal A et al., also reported high rate of isolation of bla_{CTXM} (85.4%) among ESBL isolates of north India [18]. The finding of *bla*CTX-M (10.78%) followed by bla_{TEM} (67.64%) and bla_{SHV} (15.68%) in present study was in accordance with that of an earlier study which reported 75 per cent bla_{TEM} among ESBL-producing *K. pneumoniae* isolates from Lucknow [15]. The present study showed bla_{TEM} (67.64%), and blaCTX-M (10.78%) group gene as the most prevalent in this region followed by group genes bla_{SHV} (15.68%), and bla_{OXA} (18.62%). A surveillance study in U.K showed wide scattering of CTX-M-15 producing *E. coli* [19]. In the United States TEM type beta-lactamases particularly TEM12, TEM10, TEM26 have been observed in most hospital out breaks. Recent studies of hospital associated infection showed that SHV4 and SHV5 are becoming the predominant type of ESBLs in isolates of *K. pneumoniae* [20,21]. In Germany, SHV2, and SHV5 seem to be the most predominant types and in France SHV-3, SHV-4 and TEM-3 are more common [10].

On the whole, the present study reported high prevalence of ESBL producers among cancer patients that urge the need for center-wise investigation on the ESBL surveillance to decide on the drug of choice. The presence of multiple ESBL genes in a single isolate has been reported and the same has been reflected in present study. In present study, 70% of isolates showed more than one ESBLs gene in one isolate.

Limitation(s)

Narrow geographic location is the limitation of this study.

CONCLUSION(S)

The present study showed the emergence and occurrence of ESBL producing Enterobacteriace isolates causing infection in cancer patients. All the strains isolated were mostly MDR. The presence of the ESBL genes bla_{TEM}, bla_{SHV}, bla_{CTX-M} and blocks were demonstrated from these isolates. These genes can be targeted to treat the infections caused by these resistant organisms. As cancer patients tends to suffer immune suppression, they are more prone to microbial infection than a normal individual. Hence, more scrutiny is essential while managing any infections among them especially the infections caused by ESBL producing MDR strains. Based on the authors observations, carbapenems can be considered for such therapeutic management in cancer patients.

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