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Detection of *fox-cit* gene among phenotypically confirmed amp C betalactamase producing Enterobacteriaceae in skin and soft tissue exudates in a tertiary care hospital

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Abstract
Background: Amp C β-lactamases are clinically important cephalosporinases that offers resistance to Penicillin, first, second and third generation Cephalosporin, Monobactams and β-lactamase inhibitor but susceptible to fourth generation Cephalosporins and Carbapenem antibiotics. This study aims to detect the prevalence of plasmid mediated *bla FOX-CIT* gene among phenotypically confirmed p Amp C beta lactamase producing Enterobacteriaceae in skin and soft tissue exudates in a tertiary care hospital.
Materials and Methods: A total of 685 cefotaxime resistant Enterobacteriaceae samples identified from skin and soft tissue infection by Standard Microbiological Laboratory procedures were screened for Cefoxitin resistance by disk diffusion method as per CLSI 2024 guidelines. A total 122 consecutive non-replicate cefoxitin resistant isolates were subjected to Cefoxitin-Cloxacillin double disk synergy confirmatory test (CC-DDS). Molecular genotyping by Conventional Multiplex PCR for *bla FOX-CIT* gene was done for the phenotypically confirmed isolates.
Results: Out of the total 122 Cefoxitin resistant isolates, 69(56.5%) isolates were *Escherichia coli*, 48 (39.3%) isolates were *Klebsiella pneumoniae*, 3(2.4%) isolates were *Proteus mirabilis* and 2 isolates (1.63%) were *Citrobacter koseri*. Majority of the samples were from Department of surgery. Out of those 122 isolates 55 (45.1%) isolates were positive for double disk synergy test which were then subjected to genotypic confirmation by Conventional Multiplex PCR method. Among the total 55 isolates 14 (58.3%) were detected with *FOX-CIT* gene.
Conclusion: Early and accurate detection of p Amp C β-lactamase producers through combined phenotypic and genotypic methods is vital to guide appropriate therapy and to limit the worsening of resistance. Therefore it is significant to have strict infection control measures, establishing antimicrobial stewardship and continuous surveillance in hospital facilities to prevent the inappropriate use of antibiotics and emergence of antimicrobial resistance.

Keywords: Amp C β-lactamase, cefoxitin, CC-DDS, PCR, *bla FOX-CIT*

Introduction
Antimicrobial resistance is one of the top ten public threat as per World health organisation (WHO) [1]. Individuals and healthcare systems are severely burdened by AMR in bacterial infections, which can result in longer hospital admissions, more expensive treatment, and the possibility of further transmission and greater rates of morbidity and mortality [2, 3]. The most common mechanism for antimicrobial resistance among the Gram negative bacteria is by synthesis of β-lactamases against β lactam antibiotics [4].
The Amp C β-lactamase of Escherichia coli was the first bacterial enzyme known to break down penicillin [5]. Amp C β-lactamases are belongs to the class C enzymes under the Ambler molecular classification scheme and group 1 in functional classification by Bush - Jacoby containing serine residues at their active site for catalysis [6] Amp C β-lactamases are the cephalosporinases that cause hydrolyses and inactivation of Cephamycin such as Cefoxitin, other broad-spectrum Cephalosporins, and Monobactams such as Aztreonam. They are also poorly inhibited by classical beta lactamase inhibitors such as sulbactam and clavulanic acid [7].
The mechanism of Amp C β-lactamases resistance can be divided into 3 categories which include inducible resistance via chromosomally encoded Amp C genes eg: Enterobacter cloacae, Serratia marcescens, Citrobacter freundi, Pseudomonas aeruginosa, non-inducible chromosomal resistance due to promoter or attenuator mutations eg: Escherichia coli,

Shigella species, Acinetobacter baumannii, Plasmid mediated resistance eg: *Klebsiella pneumoniae*, Escherichia coli, Salmonella species etc [8].

The plasmid-mediated Amp C genes are derived from inducible chromosomal genes that have become mobilized. Commonly reported genotypes are ACC, FOX, MOX, DHA, CIT and EBC [9]. Plasmid mediated Amp C beta lactamase are encoded on plasmids rather than the chromosome. They enable the horizontal gene transfer between the different species particularly among Escherichia coli, *Klebsiella pneumoniae*, Salmonella species. The regulatory gene Amp R keep a check on Amp β lactamase expression in chromosomal Amp C. In plasmid mediated Amp C lacks regulatory genes that leads to constitutive overexpression [10, 11].

Information regarding the presence of Amp C β-lactamases in Gram negative bacteria were minimum, as there are no established standard protocol for identifying Amp C β-lactamases producing organisms which in turn it takes a delay in choosing the appropriate antibiotics [12]. It is essential to have a knowledge about the distribution of resistant organism and antimicrobial resistance pattern of the isolates from the various departments within a hospital facility, which guide as to select the appropriate antibiotics and strengthens the infection control practices by prompt identification of resistant isolates. This study aims to detect *bla FOX-CIT* gene among the phenotypically confirmed plasmid mediated Amp C beta lactamase producing Enterobacteriaceae in skin and soft tissue exudates in a tertiary care hospital.

Materials and Methods: After obtaining Institutional Ethics Committee Clearance, this cross-sectional descriptive study was conducted in the Department of Microbiology, Stanley medical college and Hospitals in North Chennai for a period of 6 months (April 2024 -September 2024).

- **Inclusion criteria:** All aspirates and exudates samples of skin and soft tissue infections which were received in Department of Microbiology.
- **Exclusion criteria:** Repeat samples from same patients were excluded.

1. Sample collection and processing: 1.Identification of Enterobacteriaceae

During the study period, all aspirates and wound swabs of skin and soft tissue infections from various departments were processed by standard Microbiological procedures and Enterobacteriaceae were confirmed.

2. Antimicrobial susceptibility testing [13]

As per CLSI M100 2024 guidelines, Antimicrobial susceptibility testing for the isolated Enterobacteriaceae species was done by using Muller Hinton agar.

3. Amp C Screening method [14]

Isolates of E. coli, K. pneumoniae, P. mirabilis, *Citrobacter koseri* species which are resistant to cefotaxime, ceftazidime were included for Amp C beta lactamase screening by Cefoxitin disk diffusion method: Cefoxitin 30-ug disc and the isolates with zone diameters less than 18 mm were considered as Amp C producers.

2. Phenotypic Amp C confirmation method Cefoxitin Cloxacillin-Double disc synergy test [14, 15]

This test was based on the inhibitory effect of cloxacillin on Amp C production. The 122 non-replicate cefoxitin resistant isolates were subjected to Antimicrobial susceptibility testing on the Mueller Hinton agar with Cefoxitin/cloxacillin disks (200 µg/ 30 µg) and cefoxitin disk (30 µg) were used. A difference of 4 mm zone of inhibition between the two discs was considered as phenotypically confirmed of Amp C producers.

3. Genotypic detection

Conventional Multiplex PCR was done to detect the most common plasmid mediated Amp C gene bla FOX -CIT gene

PCR

PCR was performed for the bacterial isolates with 25µL volume consisting of Amp C resistant primers (Immu Genix Biosciences, India), 2X PCR Master mix, DNA template (0.1-1 µg of DNA) and PCR grade water. The PCR amplification was performed in Veriti 96-Well Thermal Cycler (Applied Biosystems, USA) with initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 30s,64°C for 30s and 72°C for 1min, and with final extension at 72°C for 7 min. The PCR were processed along with the known positive and negative control. After PCR, the amplicons were resolved along with DNA markers in 2% agarose with Ethidium bromide (10 mg/mL) by gel electrophoresis for ~20min at 135 V using Mupid-exU system (Takara, Japan) and gel was analysed by Gel documentation system (Biobee, Bangalore)

Results

The descriptive cross sectional study was conducted in the Department of Microbiology at Government Stanley Medical College and Hospital for the period of 6 months from April 2024 to September 2024. As per inclusion criteria a total 122 consecutive non-replicate cefoxitin resistant Enterobacterales from skin and soft infections were included in the study. The cefoxitin resistant isolate was identified based on the standard phenotypic identification method the study results were discussed as follows

Table 1: age -sex wise distribution of the study group (n=122)

Age in years		Male		Female	
Number of patients		Percentage (%)		Number of patients	Percentage (%)
1-9	1	0.8		0	0
10-19	5	4.1		3	2.4
20-29	14	11.5		5	4.1
30-39	6	4.9		8	6.5
40-49	15	12.3		13	10.7
50-59	23	18.9		9	7.4
60-69	13	10.7		2	1.6
>70	2	1.6		3	2.5
Total	79	64.8		43	35.2

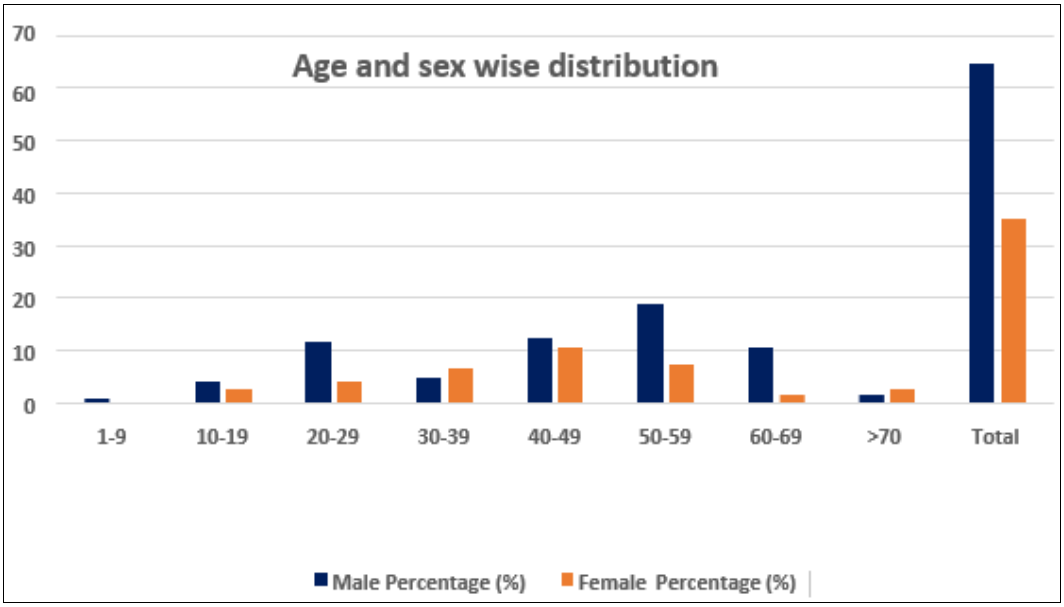

Chart 1: Age -Sex Wise Distribution of the Study Group (N=122)

Table 2: Inpatient and outpatient distribution among the study group (n=122)

In/out patient	Total samples(n=122)	Percentage (%)
Inpatient	106	86.89
Outpatient	16	13.11
Total	122	100

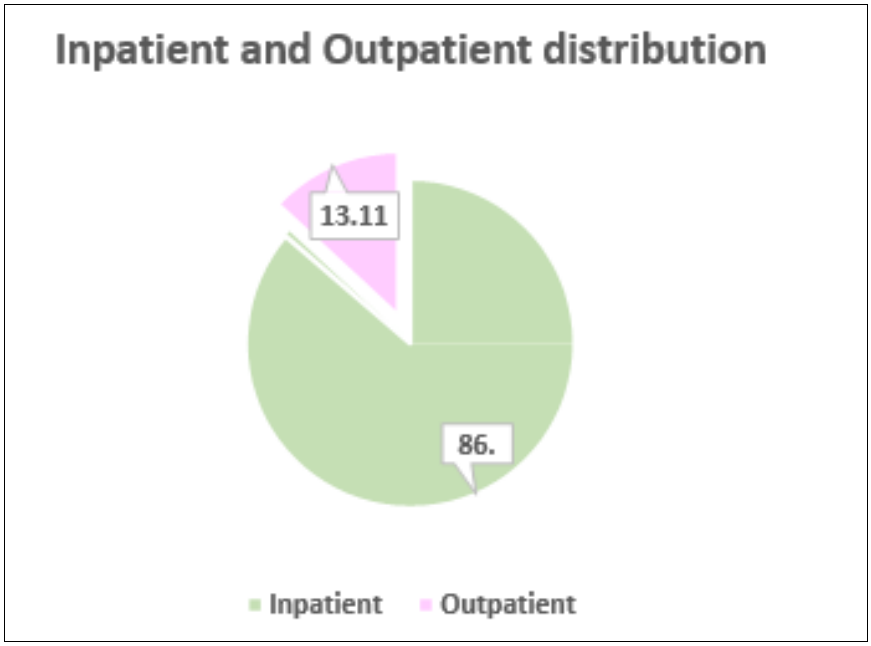

Chart 2: Inpatient and Outpatient Distribution among the Study Group (N=122)

Table 3: Ward Wise Distribution of Cefoxitin Resistant Enterobacteriaceae (N=122)

Ward	No of sample (n=122)	Percentage (%)
General surgery ward	53	43.4
Orthopaedics	35	28.7
Septic ward	16	13.1
SICU	3	2.5
Plastic surgery	3	2.5
Paediatrics	1	0.8
Others	11	9
Total	122	100

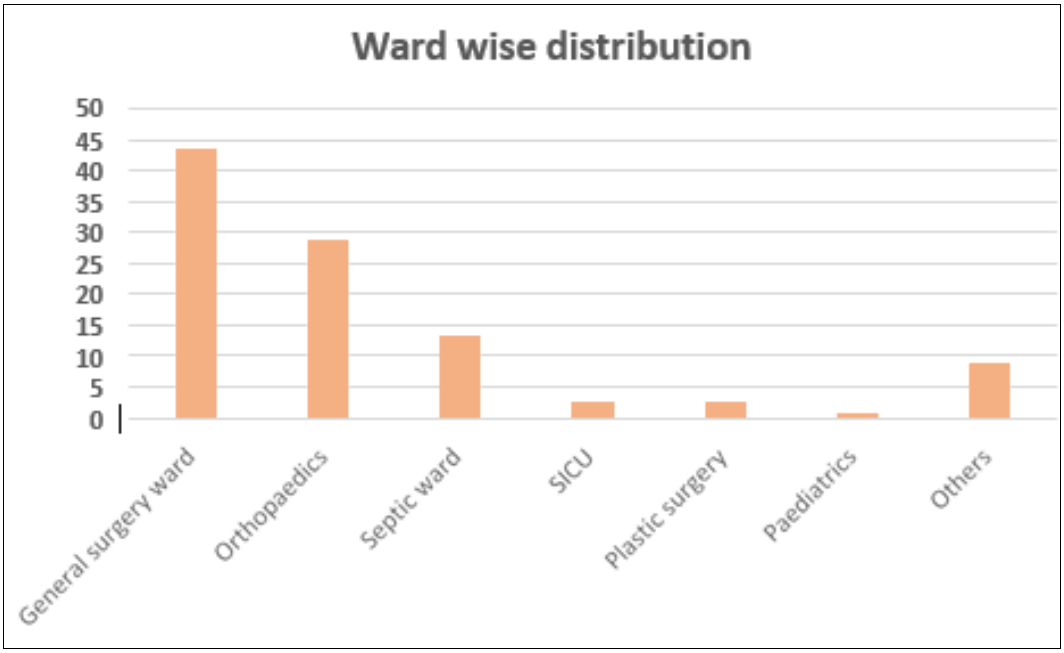

Chart 3: Ward wise distribution of Cefoxitin resistant Enterobacteriaceae (n=122)

Table 4: Distribution of Cefoxitin resistance among Enterobacteriaceae (n=122)

Organism	No of the isolates	Percentage (%)
Escherichia coli	69	56.6
Klebsiella pneumoniae	48	39.3
Proteus mirabilis	3	2.5
Citrobacter koseri	2	1.6
Total	122	100

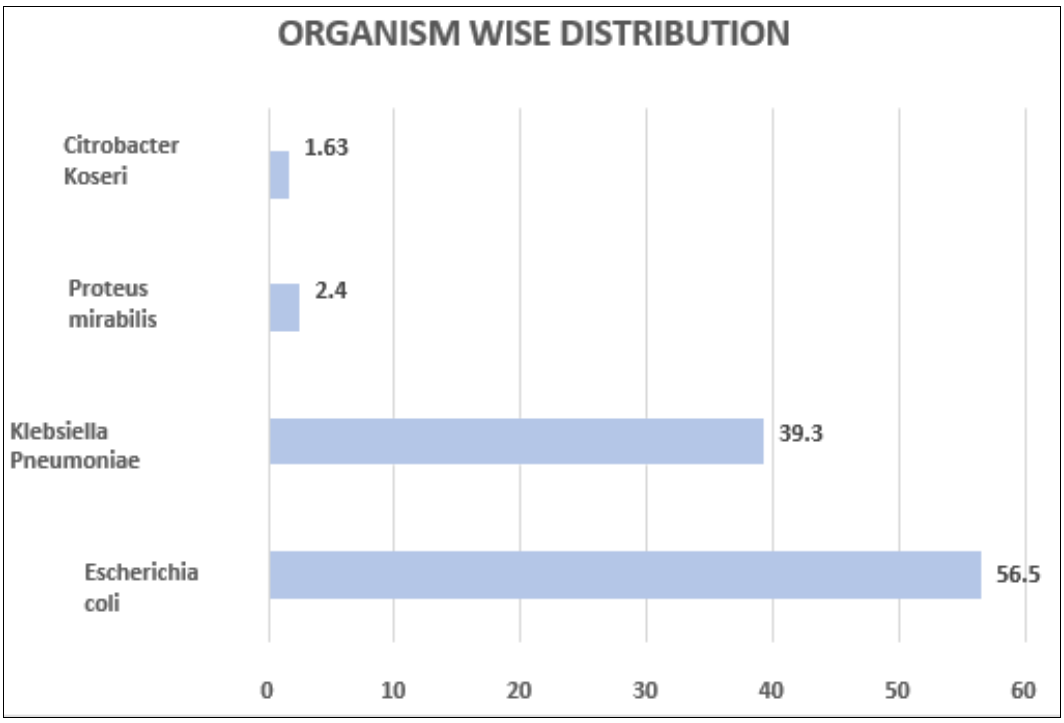

Chart 4: Distribution of Cefoxitin resistance among Enterobacteriaceae (n=122)

Table 5: Antimicrobial susceptibility pattern of Cefoxitin resistant Enterobacteriaceae (n=122)

Drug with disc content	Susceptible No. of isolates	Susceptible Percentage (%)	Resistance No. of isolates	Resistance Percentage (%)
Ampicillin 10µg	2	1.6	120	98.4
Cefotaxime 30 µg	0	0	122	100
Amoxicillin-clavulanate 20µg/10µg	12	9.8	110	90.2
Piperacillin-tazobactam	64	52.5	58	47.5

100 µg/10 µg				
Gentamicin10 µg	107	87.7	15	12.3
Ciprofloxacin 5 µg	59	48.4	63	51.6
Cotrimoxazole /23.75 µg 1.25 µg	32	26.2	90	73.8
Cefepime30 µg	119	97.5	3	2.5
Meropenem 10 µg	122	100	0	0
Amikacin 30 µg	117	95.9	5	4.1
Tetracycline 30 µg	65	53.3	57	46.7

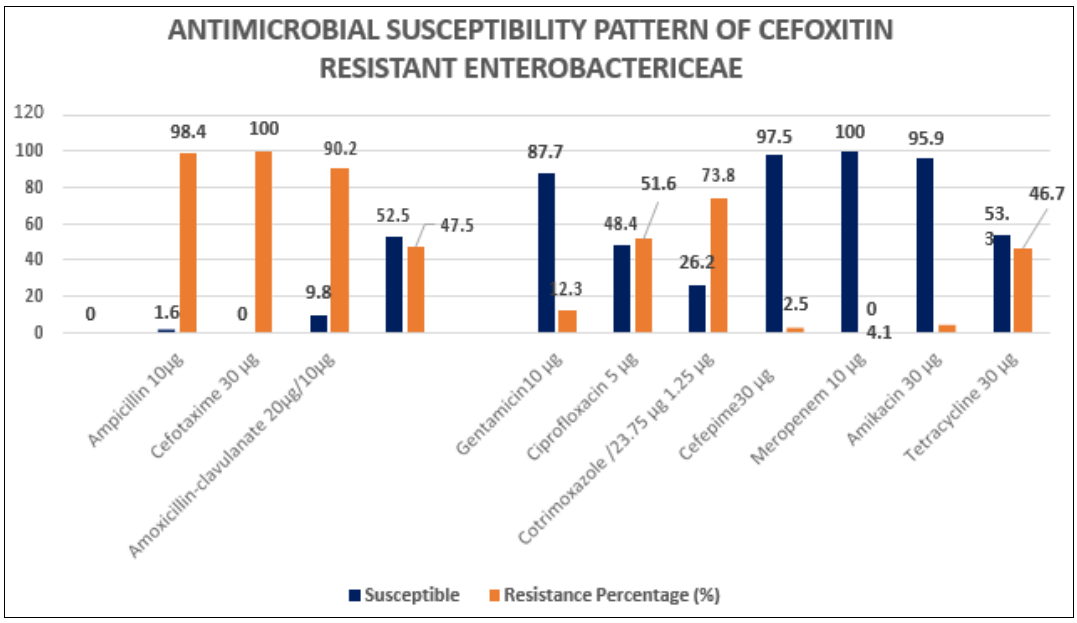

Chart 5: Antimicrobial Susceptibility Pattern of Cefoxitin Resistant Enterobacteriaceae (N=122)

Table 6: Cefoxitin Cloxacillin-Double Disc Synergy Test (Phenotypic Confirmatory Test) Among Cefoxitin Resistant Enterobacteriaceae (N=122)

Cefoxitin Cloxacillin- Double disc synergy test	No of isolates (n=122)	Percentage (%)
Positive	55	45.1
Negative	67	54.9
Total	122	100

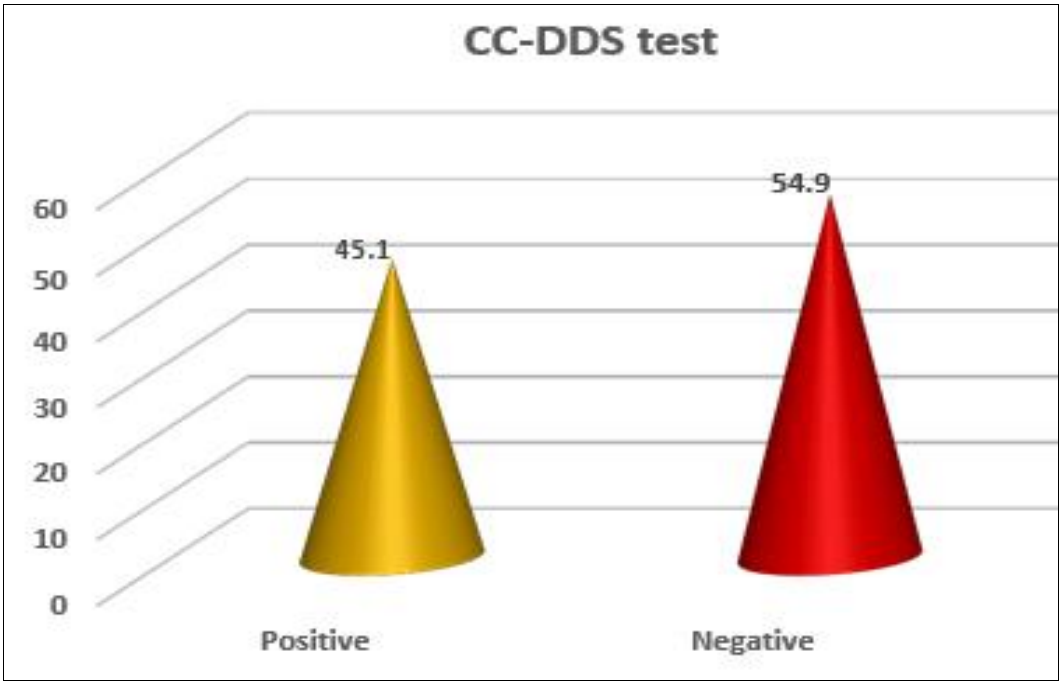

Chart 6: Cefoxitin Cloxacillin-Double Disc Synergy Test (Phenotypic Confirmatory Test) Among Cefoxitin Resistant Enterobacteriaceae (N=122)

Table 7: Distribution of Gene among Genotypically Confirmed Amp C Enterobacteriaceae (N=55)

Name of the gene	E.coli		K.pneumoniae		Total	
No of isolates		Percentage (%)	No of isolates	Percentage (%)	No of isolates	Percentage (%)
FOX	9	16.4	3	5.4	12	21.8
CIT	5	9.1	4	7.3	9	16.4
FOX-CIT	9	16.4	5	9.1	14	25.5
FOX-ACC	3	5.4	4	7.3	7	12.7
GENE NOT DETECTED	8	14.5	5	9.1	13	23.6
Total	34	61.8	21	38.2	55	100

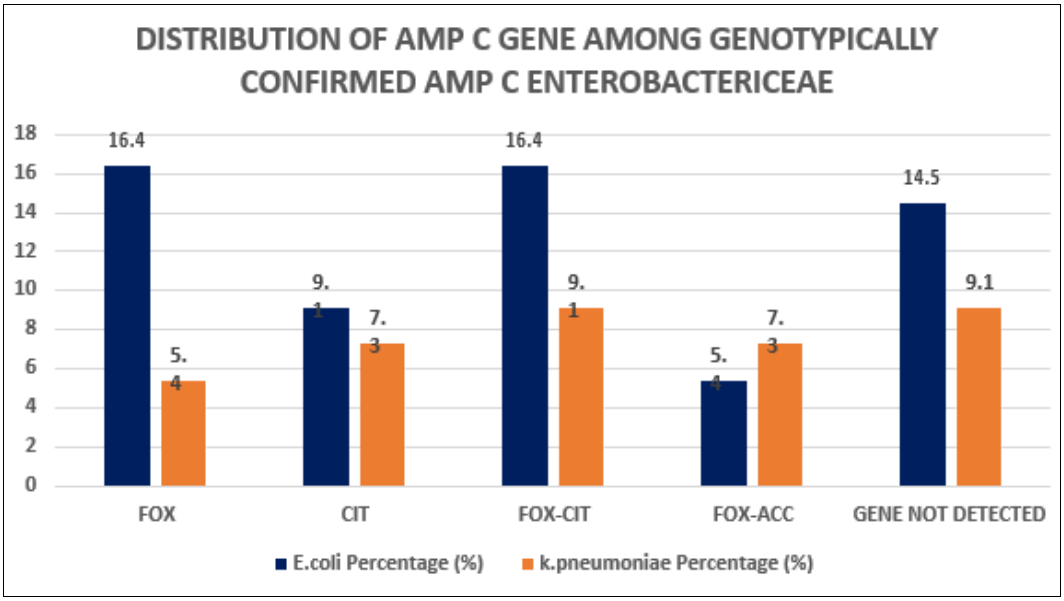

Chart 7: Distribution of Amp C Gene among Genotypically Confirmed Amp C Enterobacteriaceae (N=55)

Discussion

The increasing incidence of antimicrobial resistance remains one of the major public health problems in developing countries like India which has led to the prolonged hospital stay, increased treatment costs, constraining of therapeutic options and increased morbidity and mortality. Production of β-lactamases is the main defensive mechanism against β-lactam antibiotics. The present study was carried out to explore the presence of Amber Class C β- lactamases (Amp C) in Enterobacterales among the skin and soft tissue isolates This study further assessed the prevalence of Amp C β-lactamase gene (*bla FOX-CIT*) by Multiplex PCR assay.

A total of 122 consecutive, non-duplicate Cefoxitin resistant Enterobacterales were isolated from skin and soft tissue infection. Exudates and wound swabs received from various departments like General surgery, Orthopaedics, Septic ward, SICU, Plastic surgery, Paediatrics and others from our hospital were included in this study.

Among the 122 cefoxitin resistant Enterobacterales, majority of the sample were from male patients (64.8%) and female's accounts for (35.2%) which shows the male preponderance. [Table 1] A study conducted by Suyasha S *et al* [16] at Mumbai showed similar gender prevalence. Whereas a study done by Neetu Parmar *et al* showed female predominance (81.25%). [17] The major isolates of cefoxitin resistance were received from the patients between the age group of 50-59 years. [Table 1] This goes similar with a study conducted by Ismat *et al* which showed the highest prevalence of cefoxitin resistant isolates between the age group 51-60 years [18]

In our study majority of the cefoxitin resistant isolates were received from inpatient ward accounts for 89.9%. [Table 2] Among which most of the samples were from General surgery ward constituting 43.4% followed by Orthopaedics 28.7%, Septic ward 13.1%, SICU 2.5%,

2.5% Plastic surgery, Paediatrics 0.8% and remaining were from other department's accounts for 9%. [Table 3]

Table 4 shows that out of 122 cefoxitin resistant isolates, 69 (56.6%) were Escherichia coli, 48 (39.3%) were *Klebsiella pneumoniae*, 3(2.4%) were Proteus mirabilis and 2(1.63%) were *Citrobacter koseri*. A study conducted by Dhanushree *et al* showed similar result with Escherichia coli as the predominant isolates accounts for 76.2% followed by *Klebsiella pneumoniae* 20.1%. [14] In another study done by Mol P *et al*., it is evident that Escherichia coli 28 isolates where the most common organism followed by *Klebsiella pneumoniae*, 23 isolates Proteus species 4 isolates, *Citrobacter koseri* 2 isolates, Enterobacter 1 isolates which was consisted with my results [18].

Out of 122 cefoxitin resistant isolates, all isolates were susceptible to Meropenem 10 µg -100%, followed by Cefepime 97.5%, Amikacin 95.9%, Gentamicin 87.7%, Piperacillin-tazobactam 52.45%. Highest resistance was noted for Ampicillin 98.36% followed by Amoxicillin-clavulanic acid 90.2% and cotrimoxazole 73.8%. [Table 5] A study conducted at Amirtha Institute of Medical Science by Anand Manoharan *et al* showed susceptibility to meropenem 10 µg 97%, Amikacin 30 µg 85%, Piperacillin - tazobactam 100 µg/10 µg 74.6% which was almost consisted with my study [12].

Table 6 shows that out of 122 cefoxitin resistant

enterobacterales, 55 (45.1%) isolates were phenotypically confirmed by Cefoxitin-cloxacillin double disk synergy test for Amp C beta- lactamases. A study done by Dhanashree *et al* showed 47.5% of phenotypically confirmed Amp C isolates by Cefoxitin -cloxacillin disk synergy test which was similar to my study [14]. An another study conducted by Mubin kazi *et al* showed 55% of phenotypically confirmed Amp C isolates by Cefoxitin -cloxacillin disk synergy test. [19] The cefoxitin resistant isolates that were found to be Amp C negative in phenotypic test may due to some other factors that contribute resistance like ESBL production or may due to decreased porin channels or increased efflux pump action [20, 21]. Among the 55 phenotypically confirmed Amp C β lactamase producing Enterobacteriaceae, Amp C gene was detected in 42 (76.4%) isolates, among which 14 (25.5%) isolates were positive for *bla FOX-CIT*, 12 (21.8%) were bla FOX, 9 (16.4%) were bla CIT and 7 (12.7%) were bla FOX-ACC [Table 7].

This is similar to the study done by Mubin Kazi which showed bla FOX in 34%, bla CIT in 8%, blaDHA in 1.6%, and *bla FOX-CIT* in (30%) of isolates. In this study *bla FOX-CIT* is the most common multiple Amp C producer which is similar with our study. Manoharan *et al* showed *bla FOX-CIT* gene in 21.9% and bla EBC in 8.7% of isolates.

Among 14 *bla FOX-CIT* positive isolates, 9 (64.3%) were from Escherichia coli and 5 (35.7%) were from *Klebsiella pneumoniae* [Table 7]. Manoharan *et al* showed 43.7% of Escherichia coli with *bla FOX-CIT* gene and Bindu and Saikumar *et al* showed *bla FOX-CIT* were detected from 43.75 % of Escherichia coli and 56.25% were from *Klebsiella pneumoniae*. The different geographical regions have distinct forms of Amp C β-lactamases, according to multiple research. The difference between genotypic and phenotypic characteristics could be driven by the existence of additional Amp C β-lactamase genes that keep growing apart from the six gene families identified by conventional multiplex PCR.

Conclusion

In our study among total 685 Enterobacteriaceae 122 were screened as Cefoxitin resistant of which 55 (45.1%) were phenotypically confirmed with Cefoxitin-Cloxacillin double disk synergy test. Among the 55 phenotypically confirmed Amp C Conventional multiplex PCR showed 14 (25.5%) were detected with *bla FOX-CIT* gene. In order to effectively recognize organisms that produce Amp C, this study emphasizes the need for consistent surveillance and strong diagnostic techniques that combine phenotypic and genotypic approaches. Amp C genes are easily transferred between various bacterial species since they are plasmid-encoded (e.g., from E. coli to *Klebsiella pneumoniae*). It renders more probable for resistance to spread quickly in community and hospital settings. Multidrug resistance (MDR) is often promoted by their frequent co-location with other resistance genes (such as Carbapenems and ESBLs). Treatment options for multidrug resistant strains are limited due to paucity of newer antimicrobials in the pipeline (22) The high level of resistance to widely used antibiotics highlights the need for antimicrobial stewardship, infection control procedures. The molecular identification and early detection of Amp C β-lactamases in hospital facilities are essential for directing the right empirical treatment, preventing the spread of resistance, and enhancing therapeutic results. Every health care facilities should adhere to the Antimicrobial stewardship program (ASP) which a co-ordinated approach in healthcare settings that promotes the rational and responsible use of antimicrobials to improve patient outcomes, reduce antimicrobial resistance (AMR) and prevent the spread of drug-resistant infections.

Image 1: Cefoxitin Screening Test

Image 2: Cefoxitin - Cloxacillin Disk Synergy Test

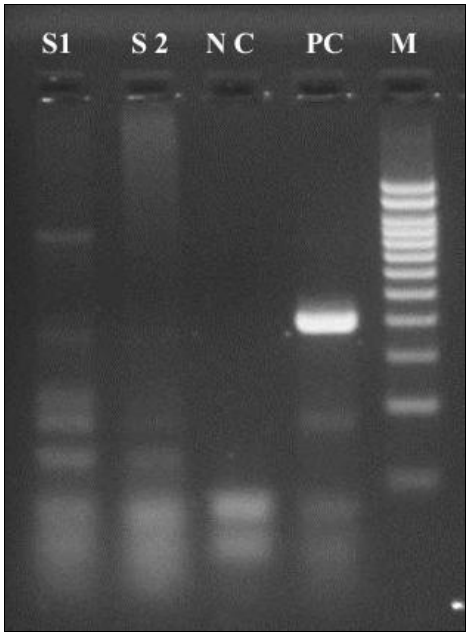

Image 3: Gel Electrophoresis for Multiplex Pcr

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